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# Genetic and evolutionary consequences of harvest in American ginseng, Panax quinquefolius L. (Araliaceae)

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# Genetic and evolutionary consequences of harvest in American ginseng, *Panax quinquefolius* L. (Araliaceae)

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**Abstract**

Genetic and evolutionary consequences of harvest in American ginseng, *Panax quinquefolius* L. (Araliaceae)

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American ginseng (*Panax quinquefolius* L.) is a wild-harvested perennial plant of the eastern deciduous forest. Harvest supplies world markets with roots used in Asian medicine, but this practice is fatal to plants. The objective of this research was to investigate the genetic and evolutionary consequences of harvest. As seen in animal species, harvest may alter size selection by preferentially removing large individuals. Chapter 2 describes my study of harvest's effects on size selection. From the simulated harvests, I observed that large-sized plants lose their fitness advantages in harvested populations. Harvest pressure could ultimately lead to population divergence if selected traits are genetically-based. As described in Chapter 3, I collected size, reproductive and age data from plants in 12 wild populations. I then used the proportion of seedlings and juvenile plants as a 'harvest index', which was based on the recovery of an experimentally-harvested population. In most study years, the age-size relationship varied with harvest index. In a separate common garden study, I also found that size differences were maintained among populations 3 to 4 years after transplantation, suggesting genetically-based variation. Harvest also reduces genetic diversity, which may lead to increased levels of inbreeding in affected populations. At the same time, unusual levels of outcrossing are possible because of 'restocking' with cultivated seeds. Chapter 4 describes the controlled crosses that I conducted to evaluate the effects of inbreeding and outcrossing with cultivated plants. The smaller size of seedlings produced from self-pollination relative to those from cross-pollination suggested inbreeding depression, but cultivated genotypes may confer accelerated growth not observed in the wild. As described in Chapter 5, I also examined the importance of genetic diversity to population growth rate. Eighteen populations were censused to obtain demographic data and their genetic diversity was assessed using neutral DNA markers (RAPD). Because of the descriptive nature of the data, I used path analysis to model and test for relationships among genetic diversity, population size and harvest pressure, and how these in turn affect population growth rate. From the results of the path analysis, harvest pressure had a negative influence on population growth, whereas genetic diversity contributed positively to population growth rates. Altogether, harvest may have far-reaching, unintended effects for populations of *P. quinquefolius* in the wild.

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# CHAPTER 1

## General Introduction

Native plant species can face a variety of anthropogenic threats. When these threats are reviewed, exploitation typically ranks below habitat destruction and invasive species in importance (Wilcove et al. 1998). However, wild harvest of plants as food, fiber or medicine is a way that humans directly interact with plant species. The use of plants as medicines is the largest use of the natural world by humans in terms of the number of species affected (Hamilton 2004). The medicinal value of a plant can have varying consequences for its conservation. In some cases, use can assign a concrete value on plant biodiversity and their habitats that can argue for their protection (Hamilton 2004, Ticktin 2004). However, use also places demands on populations in the wild, especially when harvest leads to individual mortality (Ticktin 2004).

American ginseng (*Panax quinquefolius* L.) is an herbaceous perennial plant of the eastern deciduous forest whose root is harvested for export to Asia. Harvest is fatal to the individual plant. The United States supplies an average of 60 metric tons of roots to the global market, which equates to approximately 25 million plants annually harvested from the wild (Robbins 2000). The volume of trade warranted the listing of *P. quinquefolius* in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora in 1973 (Robbins 2000). The direct effects of harvest are to reduce population size and alter population structure by removing adult plants (Lewis 1988, Van der Voort et al. 2003). Recovery of populations from harvest is limited by the slow growth of *P. quinquefolius*: as long as seven years can be needed for a seed to grow to a reproductive plant (Anderson et al. 1993). In this way, harvest can affect population growth rates to varying degrees depending on what plants are taken, if berries

are unripe or if seeds taken off site by harvesters (Van der Voort and McGraw 2006).

Harvest presents a unique situation where humans are influencing individual mortality in wild populations. If size selective, harvest could result in unintended changes to selection regimes, as documented in animal species through selective fishing gear and trophy hunting (Conover and Munch 2002, Coltman et al. 2003, Stockwell et al. 2003). Size selective harvest could be occurring in *P. quinquefolius* for several reasons. For one, most states limit harvest to the adult plants with three leaves or more (USFWS 2006). In addition, large adult plants typically yield larger, more valuable roots (Anderson et al. 1993). Harvesters may be also motivated to leave behind smaller plants to ensure future harvest (Nantel et al. 1996). Finally, large plants may simply be more apparent in the dense understory where *P. quinquefolius* grows. Given that larger plants typically have greater fitness through increased fecundity and survival rates (Carpenter and Cottam 1982, Charron and Gagnon 1991), the size-fitness relationship could be changed if large plants were preferentially targeted by harvesters. The objective of the research described in Chapter 2 was to evaluate the potential of human harvesters to alter selection on size-related traits in *P. quinquefolius*.

Human-induced evolutionary change is a conservation concern, even in long-lived species; this type of 'unnatural' selection creates the potential for species to evolve in directions unfavorable to their persistence in the wild (Ashley et al. 2003, Stockwell et al. 2003). For example, selective removal of large marine fish has resulted in earlier age at maturity and concomitant reduction in fecundity (Stockwell et al. 2003). Chapter 2

details a series of studies in which I evaluated the possible evolutionary impacts of selective harvest in *P. quinquefolius* populations. Specifically, I examined how size and reproductive traits vary with age in populations with different harvest levels. To provide an index of harvest pressure, I used the recovery pattern from an earlier study where a wild population was experimentally harvested (Van der Voort et al. 2003). A decade after harvest, the experimental population remains composed mostly of seedlings and juvenile plants. Therefore, I used the proportion of seedlings and juvenile plants as a 'harvest index' for each study population. For selective harvest to result in evolutionary change, variation in the selected trait would need to be genetically-based. I evaluated the genetic basis of size-related plant traits using a common environment approach. For this purpose, I measured plants transplanted from eight states into a forest plot originally designed as a germplasm bank. The results of the common environment study are also detailed in Chapter 3.

The consequences of harvest for genetic diversity in *P. quinquefolius* populations have been clearly defined by recent investigations. Generally, *P. quinquefolius* exhibits low levels of within-population diversity and high levels of between-population diversity (Grubbs and Case 2004, Cruse-Sanders and Hamrick 2004). Populations located in areas where harvesting is permitted have low levels of diversity relative to protected populations (Cruse-Sanders and Hamrick 2004). In addition, simulated harvests reduced diversity, even when only a small percentage of plants were removed (Cruse-Sanders et al. 2005). As in other rare or threatened plants, the low levels of diversity raise conservation concerns regarding inbreeding depression (Ellstrand and Elam 1993).

Small population size and low levels of genetic diversity can increase the frequency of inbreeding, either through increased self-pollination or mating between relatives. Inbreeding depression is a conservation concern for species without histories of close inbreeding (Charlesworth and Charlesworth 1987, Ellstrand and Elam 1993). Historical accounts of population sizes far exceed those encountered today (Lewis 1988, Charron and Gagnon 1991, Anderson et al. 1993, McGraw and Furedi 2006). At the same time, managers have encouraged the planting of cultivated seeds to 'restock' dwindling wild populations (USFWS 2006). Allozyme and randomly-amplified polymorphic DNA (RAPD) markers show that significant genetic differences exist between cultivated and wild populations (Schluter and Punja 2002, Grubbs and Case 2004). Introducing cultivated genotypes creates the potential for outbreeding depression, by which hybrids have reduced fitness (Storfer 1999). Alternatively, genes selected by cultivation techniques could confer accelerated growth, potentially allowing hybrids to outcompete native genotypes (Hufford and Mazer 2003). To evaluate the consequences of inbreeding and outcrossing with cultivated plants, I performed two sets of controlled crosses in wild populations. Three levels of inbreeding were created by crossing maternal plants with pollen from their own stamens, pollen from neighboring plants and pollen from distant plants within the population. Similarly, three levels of outcrossing were created by crossing maternal plants with pollen from other plants within the population, pollen from plants cultivated in West Virginia, and pollen from plants cultivated in Wisconsin. The offspring of these crosses were followed for four years, and the results of this study are presented in Chapter 4.

The hypothesis that genetic diversity is important to population performance and persistence underlies conservation genetics theory. In the near term, inbreeding depression or fixation of deleterious alleles through drift can impact population vital rates. Over longer time scales, variation is essential for populations to adapt to changing environmental conditions (Huenekke 1991). Nevertheless, several authors have questioned the significance of inbreeding and genetic drift when populations face more proximate threats such as habitat destruction (Lande 1988, Schemske et al. 1994, Caro and Laurenson 1994). However, results from both experimental and observational studies have supported the importance of diversity. In experimental populations of *Clarkia pulchella*, reduced genetic diversity decreased population survival probability (Newman and Pilson 1997). Plants grown from seed collected in low diversity or small-sized populations often showed reduced performance in the greenhouse or field plot (Oostermeijer et al. 1994, Heschel and Paige 1995, Fischer and Matthies 1998). Other studies of rare plants have measured the association of diversity with population performance in the wild (Menges and Dolan 1998, Schmidt and Jensen 2000, Vergeer et al. 2003, Dittbrenner et al. 2005). For example, Menges and Dolan (1998) found that genetic variation assessed by neutral markers and fire history were both strong predictors of population growth rate in *Silene regia*, a prairie perennial. Chapter 5 describes the study I performed to evaluate how genetic diversity, population size and harvest pressure relate to population growth rates in *P. quinquefolius*. In this study, I related detailed demographic data to genetic diversity estimates obtained from RAPD markers.

The four chapters each describe separate studies whose objectives were to assess the consequences of harvest for *P. quinquefolius* populations. This research provides insight relevant to the conservation and management of *P. quinquefolius* and also to the many plant species harvested from the wild worldwide.

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# CHAPTER 2

## Alteration of selection regime resulting from harvest of American ginseng, *Panax quinquefolius*[1]

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**Abstract**

Replicate harvest simulations were conducted in a large natural population of *Panax quinquefolius* L. (Araliaceae) to determine the selective effects of harvest. We investigated how minimum size requirements and the influence of size on apparency to human harvesters could result in preferential removal of large plants. To determine which plants were encountered in the large population, harvesters were tracked using GPS as they searched for every legally harvestable, adult plant they could find. Plants were assigned stage-specific fitness measures based on their contributions to population growth rate ($\lambda$) under three demographically based harvest regimes: no harvest, harvest and harvest removing seeds. Plant size was codified into a size-index equal to the product of total leaf area and stem height. Heterogeneity of slopes was tested to determine if the selection gradients ($\beta$) describing the relationship between fitness and size varied among the three harvest regimes. Harvest differentially reduced the fitness of larger plants in one of four individual harvest simulations. The combined harvest simulation significantly altered the selection regime for size in the population of juvenile and adult (harvestable) plants. Seed removal by harvesters intensified fitness declines for larger plants. Because larger plants contribute most to population growth, the selective effects of harvest could result in a shift in the evolutionary dynamics of this species with significant conservation implications.

## Introduction

Harvest can have unintended and often undesirable consequences for wild-harvested species. Harvest has been linked to local extinction, reduction in genetic diversity and altered population structure in exploited species (Ashley et al. 2003). When selective harvest targets heritable traits, harvest can lead to novel evolutionary changes in a wild species (Law 2001, Stockwell et al. 2003). The evolutionary consequences of selective harvest regimes have been illustrated in a variety of animal species, raising concerns about unintentional effects (Conover & Munch 2002, Ashley et al. 2003). Reductions in size at maturity and growth rate due to size-selective nets have been well documented for commercially exploited fish species (Miller & Kapuscinski 1994, Ratner & Lande 2001, Law 2001, Conover & Munch 2002). Trophy hunting has resulted in reduced horn size and body mass in bighorn sheep (*Ovis ovis*) (Coltman et al. 2003). Despite worldwide dependence on wild-harvested plants for food, fiber and medicine (Peters 2001, Ticktin 2004), the evolutionary consequences of harvest have not been investigated for a wild plant species (Ledig 1992, Bone & Farres 2001, Stockwell et al. 2003).

American ginseng (*Panax quinquefolius*) is an herbaceous perennial plant whose root is extensively harvested from the wild for export to the medicinal herb markets of Asia (Carlson 1986). Harvest of the root is fatal to the individual plant, and this subsequently impacts population size, structure and genetic diversity (Van der Voort et al. 2003, Cruse-Sanders et al. 2005). The scope of *P. quinquefolius* harvest is considerable, with the U.S. supplying an average of 60 metric tons (corresponding to

26.4-39.6 million plants) to the world market annually (Robbins 2000). The volume of root harvested annually prompted the Convention on International Trade in Endangered Species (CITES) to list *P. quinquefolius* on Appendix II in 1973 (Robbins 2000). Although *P. quinquefolius* has been successfully cultivated, wild harvest persists because wild roots are considered more potent in traditional Asian medicine and command nearly ten-times the price of cultivated roots (Carlson 1986, Robbins 2000).

There is preliminary, albeit circumstantial, evidence that harvest has led to a reduction in overall size of *P. quinquefolius* (McGraw 2001). In herbarium specimens collected over the 20$^{th}$ century, nine of eleven size-related traits decreased significantly while age of specimen remained consistent (McGraw 2001). Notably, this decline was most pronounced in specimens collected from Appalachian and southern states, areas that account for the largest portion of plants harvested annually (Robbins 2000, McGraw 2001). Like size reductions in other exploited species, the decline in stature of herbarium specimens of *P. quinquefolius* could be attributable to harvest if selective removal of larger individuals occurs.

Selective harvest of larger individuals could be occurring for several reasons in *P. quinquefolius*. At the time of this study, federal regulations restricted harvest to plants greater than five years in age, which can be non-destructively assessed *in situ* by counting scars on the rhizome left by the annual abscission of the stem (Anderson et al. 1993, USFWS 2002). Generally, older plants tend to be larger plants (Carpenter & Cottam 1982, Anderson et al. 1993), but regulations in most states explicitly restrict harvest to

plants with three or more leaves (USFWS 2002). Plants with larger aerial parts possess larger roots (Anderson et al. 1993), which yield higher economic returns to the harvester upon sale (Robbins 2000). Many harvesters are also motivated to ensure future harvest by intentionally leaving behind smaller reproductive plants (Nantel et al. 1996). Furthermore, larger plants could also be more apparent to harvesters in the dense forest understory where *P. quinquefolius* grows. The bright red color of the ripe berries of *P. quinquefolius* has also been noted as an important visual cue for harvesters (Hufford 1997), perhaps making larger, fecund plants especially apparent.

Generally, plant size is an important determinant of both the survival and fecundity components of fitness in natural populations (Werner & Caswell 1977, McGraw & Wulff 1983, Primack & Kang 1989). Total leaf area and stem height have been shown to be positively related to both reproductive output and year to year survival in *P. quinquefolius* as well (Carpenter & Cottam 1982, Lewis & Zenger 1982, Charron & Gagnon 1991, Anderson et al. 1993). However, this dependence of fitness on plant size could be altered if selective harvest preferentially removed larger individuals. Harvesters may also commonly remove seeds present on harvested plants, which could further impact plant fitness (Cruse-Sanders et al. 2005). Anecdotal evidence suggests that seeds are likely planted in plots near the harvester's home for later extraction (USFWS 2002). Because laws in most states require seeds to be planted on site (USFWS 2002), the fate of seeds after removal is difficult to determine from harvester interviews as this would constitute illegal behavior (Bailey 1999). Nevertheless, because harvest of *P. quinquefolius* can directly target the essential components of fitness—survival and

reproduction—size-selective harvest could result in novel evolutionary changes in this species.

The objective of this study was to determine if *P. quinquefolius* harvest results in alteration of the selection regime for plant size. We hypothesized that selection against larger plants could occur in the harvest simulations through the influence of minimum size restrictions or the effect of size on apparency to human harvesters. Using harvest simulations, we determined how the relationship between fitness and plant size differs in a natural population experiencing three demographically based harvest regimes: no harvest, harvest and harvest taking seeds. Alteration of the existing relationship between fitness and plant size could predict the magnitude and direction of possible evolutionary changes in *P. quinquefolius* as a result of harvest.

## Methods

*Study Species Natural History*

American ginseng, *Panax quinquefolius* L. (Araliaceae), is a long-lived perennial herb that grows in the understory of the eastern deciduous forest. Once common, the decline of wild populations has been linked to overharvesting, increased browse by overabundant white-tailed deer, habitat loss and degradation. Plants consist of a central stem and one to four (rarely more) palmately compound leaves. Individuals are readily classified into five stage classes: seeds, seedlings (1-leaf plants), juveniles (2-leaf plants), small adults (3-leaf plants with <250 $cm^2$ total leaf area), and large adults (3-leaf with >250 $cm^2$ total leaf area and 4-leaf plants) (McGraw & Furedi 2005; Figure 2.1). Plants

with two or more leaves produce an inflorescence of self-compatible flowers in mid summer (Lewis & Zenger 1983, Schlessman 1985). Flowers yield 1-3 seeded berries in late summer, which then ripen to a characteristic red color in early autumn (McGraw et al. 2005). Seeds of *P. quinquefolius* exhibit morphophysiological dormancy and remain in the soil for at least 18-22 months before germination (Anderson et al. 1993, Baskin & Baskin 1998). The persistent soil seed bank is critical for populations to recover from harvest (Lewis 1988, Van der Voort et al. 2003).

*Study Population and Plant Traits*

The harvest simulations took place in a large population of *P. quinquefolius* located in a mixed deciduous forest near Morgantown, WV. The population consisted of 391 plants of all stage classes widely distributed across approximately 2 hectares. As part of an ongoing monitoring effort, each plant in the study population was cryptically labeled with an aluminum nail engraved with a unique number. Individual plants were relocated using a system of 'phototrails', which combine distances and angles with digital photographs. In May 2003, phenotypic traits were measured on each plant, including stem height, leaf number, length and width of longest leaflet per leaf. Total leaf area of each individual was determined using regression equations relating leaf number and leaflet measurements to observed leaf areas (McGraw & Furedi 2005).

Prior to the harvest simulations in August 2003, the locations of individual plants were mapped by marking each plant or cluster (plants within 5m of each other) with a unique GPS point using a Garmin GPS V (Garmin International, Ltd. 2003). Plants that

had their aboveground portion deer-browsed prior to harvest simulations were not included in the selection analyses. A total of 135 legally harvestable adult plants remained in the population, with the plants being approximately evenly divided among the large and small adult stage classes.

*Harvest Simulations*

The individual harvest simulations took place in late August and early September 2003, which coincided with the early part of the harvest season in West Virginia beginning August 15, 2003. Because the exceptional size of the study population warranted its protection, four volunteer 'harvesters' were recruited who could readily identify *P. quinquefolius*, but who could be trusted to keep the location of the population confidential. Flags were placed 20m from the last known plant on all sides of the population forming a rectangular area of 150m X 200m; this demarcation was necessary because the harvesters had no prior experience with the population. Beginning at the same location, each harvester was given two hours to search the area unaccompanied. To test for the influence of minimum size restrictions, the harvesters were informed of state harvest criteria and asked to comply with them. Although federal law at the time of this study allowed harvest of any size of plant greater than 5 years of age, harvest is limited to plants with three or more leaves in West Virginia (USFWS 2002). The harvesters were instructed to 'harvest' all legally harvestable plants they could find by placing a flag next to the plant, but not actually digging the plant. At the end of the search period, the harvested plants were recorded and all flagging was removed. The sparse understory vegetation allowed for no trace of a previous harvester's path to remain at the site.

While searching, each harvester carried a GPS unit (Garmin GPS V, Garmin International, Inc. 2003) that recorded their location every three seconds. Following each harvest simulation, all plants were classified into one of three categories: encountered (harvested), encountered (not harvested) and not encountered. Whether or not a plant was encountered was determined by overlaying each harvester's track with the map of the plants using Erdas Imagine GIS software (Leica Geosystems 1999; Figure 2.2). Plants beyond 6m of the track were considered not encountered. We determined the threshold distance by comparing the distances between harvester tracks to harvested plants; based on an average distance of approximately 4.5m, the threshold distance of 6m would conservatively classify plants as not encountered. These data were used to analyze selection in alternate ways (see below).

The overall efficiency or rate of harvest was determined as the number of harvestable adult plants each harvester flagged during their two-hour searches. The search efficiency of each harvester was quantified as the percentage of plants that were harvested, of those that were encountered. Differences in efficiencies between harvesters were tested using a log-likelihood test (Sokal & Rohlf 1995) in SAS JMP v. 5.1 statistical software (SAS Institute Inc. 2002).

*Estimating Fitness*

Because harvesters are unlikely to find or remove all plants (Lewis 1988, Van der Voort et al. 2003), harvest acts as an event of differential mortality in a population.

Measuring fitness based on one period of differential mortality can yield important information about selection acting on phenotypic variation (Lande & Arnold 1983, Janzen & Stern 1998). However, fitness estimates based on mortality alone are insufficient because reproduction is not considered. Measuring an individual's contribution to population growth is a more biologically relevant fitness estimate because it incorporates both survival and fecundity (McGraw & Caswell 1996).

To estimate stage-specific plant fitness, we used the sample influence function ($SIF_i$) measured as the contribution of each individual *i* to the stable population growth rate ($\lambda$) (McGraw 1989, Vavrek et al. 1996, Vavrek et al. 1997). The stable population growth rate ($\lambda$), is the dominant eigenvalue for the transition probability matrix **A** (Caswell 2001). $SIF_i$ was calculated by first removing individual *i* from the population, and then re-estimating the stable population growth rate ($\lambda_{-i}$). The contribution to $\lambda$ of each individual was then calculated using:

$$\phi_i = n\lambda - (n-1)\lambda_{-i}$$

where n is the number of individuals in the population. The sample influence function is then calculated by:

$$SIF_i = \phi_i - \bar{\phi}$$

$SIF_i$ provides an estimate of stage-specific fitness by incorporating the overall effect of an individual's survival, growth and reproduction on population growth; thus, a positive $SIF_i$ value means that the individual's behavior is having a positive effect on $\lambda$ and *vice versa* for a negative $SIF_i$ value (McGraw 1989, Vavrek et al.1996).

The transition probability matrix (***A***) was constructed based on transitions between stage classes observed from May 2003 to May 2004. From one year to the next an individual may grow (advance in stage), stay at the same stage, regress (decline in stage), reproduce or die (Figure 2.1). The study population was censused in May of 2003 and all individuals were assigned to stage classes based on leaf number and area as above. The plants were again visited in August of 2003 to record seed production. In May of 2004, the population was censused again to observe the year to year transitions plus the appearance of new seedlings. The small percentage of plants present in 2003 but not in May of 2004 were assigned fates according to the protocols used in prior demographic studies of this species (McGraw & Furedi 2005). Seed bank dynamics were estimated from experimental seed cages planted at the population in 2002 (McGraw & Furedi 2005).

In addition to the observed 2003-2004 transition matrix (no harvest), each of the four harvest simulations produced two alternative transition matrices assuming two levels of harvest (harvest, harvest taking seeds). To produce the transition matrix for the first level of harvest, plants taken by harvesters were assigned a status of 'death' in 2004, since harvest is fatal to plants. For the second level of harvest, the harvested plants died and did not contribute seeds in 2003, simulating removal of seeds from the population in the transition matrix. The individual harvest simulations were also pooled to create a combined harvest simulation to represent the cumulative effect of the separate 2-hour searches. In the combined harvest simulation matrix, a plant was assigned a fate of death if removed by any of the four harvesters. Three separate fitness measures were

calculated for each individual as their contributions to population growth rate ($SIF_i$) under three harvest regimes (no harvest, harvest, and harvest taking seeds) for each harvest simulation and for the combined harvest simulation. Calculations of stage-specific fitness and matrix manipulations were performed using MATLAB version 4.0 (MathWorks 1993).

*Selection Analyses*

Consistent with other studies of selection operating in plant populations (Kalisz 1986, Kelly 1992, Bennington & McGraw 1995), we used a regression-based approach to determine the magnitude and direction of selection (Lande & Arnold 1983, Janzen & Stern 1998). To avoid difficulties in multiple-regression analysis resulting from highly interdependent traits (Mitchell-Olds & Shaw 1987), a single size index was calculated for each individual by taking the product of 2003 stem height and total leaf area. Size index was transformed to a standardized measure in units of standard deviations for use in the selection analyses (Lande & Arnold 1983). Outliers in the distribution of standardized size indexes were identified using Mahalanobis distances in SAS JMP v. 5.1 statistical software (SAS Institute Inc. 2002); two exceptionally large plants (having Mahalanobis distances >4.9) were eliminated from the subsequent selection analyses.

Two separate sets of selection analyses were performed on different subsets of the overall population: one including all juvenile and adult plants, and one including only encountered adult plants. Among encountered adult plants, changes in the patterns of selection would reflect the influence of the differential apparency of larger plants to

harvesters or microsite effects. Specifically, adult plants not encountered by harvesters in their extensive searches may predominantly occur in difficult to access microsites (e.g. on top of a rock or under dense shrub cover). In all juvenile and adult plants, changes to the patterns of selection would occur through the combined influences of microsite, harvesting regulations and plant apparency to harvesters. Although juvenile plants are not legally harvestable, they are capable of reproduction and may have differential fitness with and without harvest.

Linear selection gradients ($\beta$) were determined from the regression of stage-specific fitness on standardized size indexes of plants under each harvest regime (no harvest, harvest and harvest taking seeds). Linear selection gradients estimate the strength and direction of selection (Lande & Arnold 1983). We then used an analysis of covariance (ANCOVA) testing for heterogeneity of slopes to compare selection gradients among the three harvest regimes. Tests for variation in selection patterns were performed for each of the four harvest simulations and for the combined harvest simulation. The interaction term (Harvest Regime X Size Index) was used to determine if the selection pattern varied among harvest regimes. Non-linear models of the relationship between fitness and size-index were also analyzed for each harvest simulation to test for non-linear (stabilizing or disruptive) selection. Significance of the interaction term (Harvest Regime X Size Index) determined if the pattern of non-linear selection varied among harvest regimes. All statistical analyses were performed using SAS JMP v. 5.1 statistical software (SAS Institute Inc. 2002).

## Results

*Harvest Simulations*

The overall search efficiency—the number of adult plants removed during the two hour search—varied among individual harvesters (likelihood ratio = 16.772, p = 0.0008). The number of plants harvested during each harvest simulation ranged from 16.3% to 36.3% of the 135 adult plants in the population (Table 2.1). In accordance with harvest regulations, no juvenile plants were harvested during any of the simulations. However, many legally harvestable plants were never encountered during the harvest simulations. When only the encountered plants are considered, the efficiency of harvest increased, ranging from 39.4% to 57.6% of adult plants removed (Table 2.1), and the search efficiencies no longer varied among harvesters (likelihood ratio = 6.725, p = 0.0812).

*Selection- All Juveniles and Adults*

In the population of both juvenile and adult plants without harvest, a significant positive slope of the linear selection gradient ($\beta$ = 0.1450) indicated that larger plants had higher stage specific fitness than smaller plants (Table 2.2). There was no evidence for significant non-linear selection in the population of juvenile and adult plants ($\gamma$ = - 0.0072; t = - 0.22, $p$ = 0.8261).

When the population experienced harvest or harvest taking seeds, the slope of the linear selection gradients did not decline significantly in any of the individual harvest simulations (Figure 2.3, Table 2.3). However, in the combined harvest simulation, the slope of the linear selection gradient declined significantly as the population was

harvested (F = 3.7797, $p$ = 0.0233). Removal of seeds during the combined harvest further decreased the selection gradient such that the slope was not significantly different than zero ($\beta$ = 0.0138; t = 0.45, $p$=0.6519). Variation in non-linear selection was not detected in any harvest simulation or in the combined harvest simulation.

*Selection-Encountered Adults*

In the population without harvest, the slope of the selection gradients ($\beta$) ranged from 0.1489 to 0.1958 for the encountered adult plants, again indicating a positive relationship between size-index and stage-specific fitness. In harvest simulations 2 and 4, these gradients were statistically significant and the same trends were found in simulations 1 and 3 (Table 2.4). Also in the combined harvest simulation, there was a positive relationship between size-index and stage-specific fitness among encountered adult plants ($\beta$ = 0.1738; t = 2.98, $p$ = 0.0035).

Harvest significantly reduced the slope of the selection gradients describing the relationship between size-index and stage-specific fitness for harvest simulation 2, and again this decline was exacerbated when harvesters removed seeds (Table 2.5, Figure 2.4). A similar trend ($0.05 < p < 0.10$) towards an altered pattern of selection was also observed for harvest simulation 3, and this was also significant for the combined harvest simulation.

## Discussion

The evidence for selection favoring large individuals in the population without harvest is in accord with previous observations of the life history of *P. quinquefolius*. The components of the size-index (total leaf area and stem height) are known to be significant positive predictors of both seed production and year to year survival in *P. quinquefolius* (Carpenter & Cottam 1982, Lewis & Zenger 1982, Charron & Gagnon 1991, Anderson et al. 1993). The magnitudes were also consistent with the median of published standardized selection gradients (0.16) reported for quantitative traits in other wild species (Kingsolver et al. 2001). The selection regimes operating in all juveniles and adults as a whole, and encountered adults in this population were all remarkably similar. Previous studies of the variation in selection across life cycle stages have found gradients to mostly vary in magnitude (Kelly 1992); however measurement of selection at later life stages can be dramatically influenced by previous episodes of differential mortality (Bennington & McGraw 1995).

Individual harvesters did not significantly affect the selection regime present in the population of juvenile and adult plants. Nevertheless, the direction of the change was consistent across harvest simulations, suggesting insufficient numbers of harvested plants to detect differences. Corroborating this was the observation that the fitness advantages of larger plants declined significantly in the combined harvest simulation. Although reported rates of harvest are limited, the combined harvest simulation may be more realistic for several reasons. In one published observation of harvest, diggers were able to remove all but one plant they found in the population (Lewis 1988). High prices can

encourage harvesters to allocate more time to searching (Bailey 1999), likely more than the two hours used in this study. The size of the study population meant that large proportions of plants were never encountered, thus diluting the overall effects of individual harvesters. Given the above issues, the results of this study do not exclude the contribution of legal minimum size restrictions to selective harvest of *P. quinquefolius*.

The most prominent declines in the slopes of linear selection gradients in the individual harvest simulations occurred in the population of encountered adult plants. The removal of seeds exacerbated the selective effects of harvest by further decreasing the fitness advantages of larger plants. Although the decline of slopes was consistent across simulations, the small number of adults removed may again explain the lack of widespread significance for these declines. Nonetheless, in at least one harvest simulation, selective harvest resulted from the differential removal of large adults that were encountered. This result suggests that the selective harvest of large plants was influenced by the contribution of size to apparency. Ripe, red berries are a noted visual cue for harvesters (Hufford 1997), which could additionally heighten the increased apparency of larger, more fecund plants. Although few berries were ripe at the time of the harvest simulations, this is realistic as start of legal harvest season in the majority of states occurs before berry ripening (McGraw et al. 2005). Harvests occurring when berries are ripe would be expected to strengthen our conclusion that the size-fitness relationship is altered by harvest.

Selection operating through apparency to human harvesters is most equivalent to studies of evolution of weed species in response to weeding pressures (Barrett 1983, Kadereit & Briggs 1985, Briggs et al. 1992). Like the selection observed in this study, selection imposed by weeding results from unintentionally selective mortality. Unlike typical artificial selection, changes effected by unintentional selection often occur in unanticipated or unwanted directions from the perspective of the human selective agent. For example, hand weeding of rice fields resulted in *Echinochloa crus-galli* genotypes that mimic shoots of crop seedlings and 'escape' removal (Barrett 1983). Similarly, unintentional selection created by preferentially removing larger ginseng plants would favor smaller plants, perhaps with less valuable roots.

In contrast to unintentional selection, selective harvest regimes in wild harvested animal species have generally resulted from intentional size-selectivity to increase economic yield or comply with well-intentioned conservation regulations (Rowell et al. 1989, Miller & Kapuscinski 1994, Ratner & Lande 2001, Conover & Munch 2002). Selective harvest of plants is perhaps most similar to the practice of 'high-grading' in commercial timbering, which has been speculatively linked to changes in exploited tree species in temperate and tropical forests (Ledig 1992). Intentionally selective harvest could also be a factor during wild harvest of *P. quinquefolius*. Harvesters could be motivated to deliberately leave small harvestable-sized plants behind for several reasons; for example, diminishing economic returns for the time invested in freeing relatively small roots from the soil. Systematically leaving behind small plants in combination with

minimum size requirements and the influence of apparency could actually increase the fitness of small plants relative to that of large plants.

Although this study reports evidence for size-selective harvest, many other factors are important to consider before harvest can be considered a force in the evolution of *P. quinquefolius* populations. Namely, variation in total leaf area and stem height must be related to underlying heritable variation. Furthermore, harvest of *P. quinquefolius* is also subject to the 'tragedy of the commons' such that harvesters are motivated to leave no plants behind in a population, regardless of size or existing laws. This intensity of harvest can threaten population persistence (Nantel et al. 1996, Van der Voort et al. 2003), and thus population extinction could preclude a response to selection. The ability of a wild population to respond to anthropogenic selective pressures is ultimately a function of both genetic and demographic factors and can be difficult to predict (Burger & Lynch 1995, Gomulkiewicz & Holt 1995). Nevertheless, the decrease in the fitness advantage of larger plants has the potential to alter the size distribution in harvested populations.

In a long-lived perennial plant such as *P. quinquefolius*, reduced or lost selective advantage may also affect traits beyond those directly associated with size. Studies in other plant species have illustrated the ability of selection on a specific character to influence other traits or life-history events through phenotypic or genetic correlations (Kalisz 1986, Kelly 1992, Bennington & McGraw 1995). In harvested populations of *P. quinquefolius*, growth to larger size classes may no longer be favored, as allocation to growth would come at additional costs to survival and/or reproductive output. Because

larger plants contribute disproportionately to population growth, smaller, less fecund plants would have additional consequences for population persistence. More complex indirect effects may be important as well in harvested populations. Altered fitness costs for reproduction at later stages may favor enhanced seed production by reproductive juvenile plants, which typically act only as pollen donors in the population (Schlessman 1987). Some evidence exists for this type of life-history change; in a study of 21 populations in four states, a higher proportion of juvenile plants were reproductive in unprotected populations versus protected populations (Cruse-Sanders & Hamrick 2004). Altogether, these examples illustrate the potentially far-reaching effects of human interaction with natural populations. Alteration of selection patterns due to harvest as observed in this study could result in a significant shift in the evolutionary dynamics for this species.

Table 2.1: Number of small and large (total leaf area >250cm$^2$) adult plants in the population that were encountered and harvested, encountered but not harvested, and not encountered by the harvester (>6m from harvester track).

| *Harvest Simulation* | *Encountered (Harvested)* | | *Encountered (Not Harvested)* | | *Not Encountered* | *Search Efficiency*[a] *(%)* | *Overall Efficiency*[b] *(%)* |
|---|---|---|---|---|---|---|---|
| | *Small* | *Large* | *Small* | *Large* | | | |
| 1 | 14 | 14 | 21 | 22 | 64 | 39.4 | 20.7 |
| 2 | 17 | 22 | 29 | 26 | 41 | 41.5 | 28.9 |
| 3 | 10 | 12 | 13 | 15 | 85 | 44.0 | 16.3 |
| 4 | 27 | 22 | 16 | 20 | 50 | 57.6 | 36.3 |
| Combined | 39 | 41 | 22 | 16 | 17 | 67.8 | 59.3 |

[a]Search efficiency is the percent of encountered plants harvested.
[b]Overall efficiency is the percentage of plants harvested of all adults in the population.

Table 2.2: Slopes of linear selection gradients (β) and their standard errors for the relationship between stage-specific fitness and standardized size-index of juvenile (2-leaf) and adult (3 and 4-leaf) plants across harvest simulations.

| *HARVEST SIMULATION* | *SIZE INDEX* | | | | |
|---|---|---|---|---|---|
| | *Harvest Regime* | *β* | SE | *t* | *p*-value |
| | No harvest | 0.1450 | 0.0440 | 3.30 | 0.0011 |
| 1 | Harvest | 0.0833 | 0.0362 | 2.30 | 0.0223 |
| | Harvest taking seeds | 0.0681 | 0.0363 | 1.88 | 0.0618 |
| 2 | Harvest | 0.0728 | 0.0339 | 2.15 | 0.0327 |
| | Harvest taking seeds | 0.0400 | 0.0338 | 1.18 | 0.2375 |
| 3 | Harvest | 0.0588 | 0.0372 | 1.58 | 0.1150 |
| | Harvest taking seeds | 0.0326 | 0.0374 | 0.87 | 0.3839 |
| 4 | Harvest | 0.0924 | 0.0340 | 2.72 | 0.0071 |
| | Harvest taking seeds | 0.0848 | 0.0342 | 2.48 | 0.0138 |
| Combined | Harvest | 0.0418 | 0.0301 | 1.39 | 0.1668 |
| | Harvest taking seeds | 0.0138 | 0.0306 | 0.45 | 0.6519 |

Table 2.3: Results of ANCOVA testing for heterogeneity of slopes among three harvest regimes in the population (no harvest, harvest, and harvest taking seeds) for the relationship of stage-specific fitness to size-index of both juvenile and adult plants

| *HARVEST SIMULATION* | *Source* | *df* | *MS* | *F* | *p-value* |
|---|---|---|---|---|---|
| 1 | Harvest Regime (HR) | 2 | 0.0301 | 0.0809 | 0.9223 |
| | Size-Index (SI) | 1 | 7.1520 | 19.2556 | <0.0001 |
| | HR X SI | 2 | 0.4037 | 1.0870 | 0.3378 |
| | Error | 729 | 0.3714 | | |
| 2 | Harvest Regime (HR) | 2 | 0.0276 | 0.0802 | 0.9230 |
| | Size-Index (SI) | 1 | 5.4134 | 15.7266 | <0.0001 |
| | HR X SI | 2 | 0.7025 | 2.0408 | 0.1307 |
| | Error | 729 | 0.3442 | | |
| 3 | Harvest Regime (HR) | 2 | 0.0192 | 0.0501 | 0.9511 |
| | Size-Index (SI) | 1 | 4.5503 | 11.8518 | 0.0006 |
| | Regime X SI | 2 | 0.8427 | 2.1949 | 0.1121 |
| | Error | 729 | 0.3839 | | |
| 4 | Harvest Regime (HR) | 2 | 0.0391 | 0.1127 | 0.8935 |
| | Size-Index (SI) | 1 | 8.4533 | 24.3810 | <0.0001 |
| | HR X SI | 2 | 0.2613 | 0.7536 | 0.4710 |
| | Error | 729 | 0.3467 | | |
| Combined | Harvest Regime (HR) | 2 | 0.0174 | 0.0566 | 0.9450 |
| | Size-Index (SI) | 1 | 3.2763 | 10.6405 | 0.0012 |
| | HR X SI | 2 | 1.1637 | 3.7794 | 0.0233 |
| | Error | 729 | 0.3079 | | |

Table 2.4: Slopes of linear selection gradients (β) and their standard errors for the relationship between stage-specific fitness and size-index of encountered adult plants among three harvest regimes (no harvest, harvest and harvest taking seeds).

| *HARVEST SIMULATION* | *SIZE INDEX* | | | | |
|---|---|---|---|---|---|
| | *Harvest Regime* | *β* | *SE* | *t* | *p-value* |
| 1 | No harvest | 0.1510 | 0.0835 | 1.81 | 0.0749 |
| | Harvest | 0.0942 | 0.0535 | 1.76 | 0.0828 |
| | Harvest taking seeds | 0.0497 | 0.0575 | 0.87 | 0.3900 |
| 2 | No harvest | 0.1958 | 0.0705 | 2.78 | 0.0067 |
| | Harvest | 0.0585 | 0.0434 | 1.35 | 0.1809 |
| | Harvest taking seeds | 0.0126 | 0.0441 | 0.29 | 0.7757 |
| 3 | No harvest | 0.1489 | 0.0891 | 1.67 | 0.1013 |
| | Harvest | -0.0075 | 0.0457 | -0.12 | 0.9080 |
| | Harvest taking seeds | -0.0764 | 0.0691 | -1.11 | 0.2742 |
| 4 | No harvest | 0.1860 | 0.0737 | 2.52 | 0.0136 |
| | Harvest | 0.1317 | 0.0485 | 2.71 | 0.0081 |
| | Harvest taking seeds | 0.1168 | 0.0500 | 2.34 | 0.0219 |
| Combined | No harvest | 0.1738 | 0.0584 | 2.98 | 0.0035 |
| | Harvest | 0.0174 | 0.0242 | 0.72 | 0.4747 |
| | Harvest taking seeds | 0.0216 | 0.0228 | 0.95 | 0.3499 |

Table 2.5: Results of ANCOVA testing for heterogeneity of slopes among three harvest regimes (no harvest, harvest, and harvest taking seeds) of the relationship of stage-specific fitness to size-index of encountered adult plants.

| *HARVEST SIMULATION* | *Source* | *df* | *MS* | *F* | *p-value* |
|---|---|---|---|---|---|
| 1 | Harvest Regime (HR) | 2 | 0.9472 | 3.1046 | 0.0470 |
| | Size-Index (SI) | 1 | 2.0200 | 6.6207 | 0.0108 |
| | HR X SI | 2 | 0.1794 | 0.5879 | 0.5564 |
| | Error | 204 | 0.3051 | | |
| 2 | Harvest Regime (HR) | 2 | 0.4074 | 1.4992 | 0.2251 |
| | Size-Index (SI) | 1 | 2.2000 | 8.0962 | 0.0048 |
| | HR X SI | 2 | 0.8415 | 3.0967 | 0.0468 |
| | Error | 273 | 0.2717 | | |
| 3 | Harvest Regime (HR) | 2 | 0.0698 | 0.2498 | 0.6180 |
| | Size-Index (SI) | 1 | 1.5683 | 5.6100 | 0.0045 |
| | HR X SI | 2 | 0.6619 | 2.3676 | 0.0973 |
| | Error | 144 | 0.2796 | | |
| 4 | Harvest Regime (HR) | 2 | 0.7390 | 2.5422 | 0.0807 |
| | Size-Index (SI) | 1 | 5.3306 | 18.3389 | <0.0001 |
| | HR X SI | 2 | 0.1123 | 0.3864 | 0.6799 |
| | Error | 249 | 0.2907 | | |
| Combined | Harvest Regime (HR) | 2 | 0.2108 | 1.1249 | 0.3258 |
| | Size-Index (SI) | 1 | 1.8813 | 10.0387 | 0.0017 |
| | HR X SI | 2 | 0.9906 | 5.2858 | 0.0055 |
| | Error | 369 | 0.1874 | | |

Figure 2.1: Life-cycle diagram for *Panax quinquefolius* showing typical yearly (May to May) transitions in this species

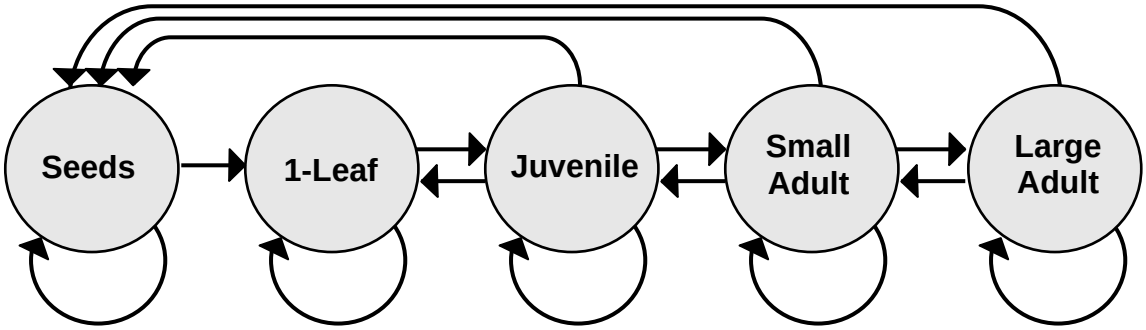


Figure 2.2: Portion of harvester track overlaid with a map of plants created using GPS points displayed in Erdas Imagine GIS software (Leica Geosystems 1999)

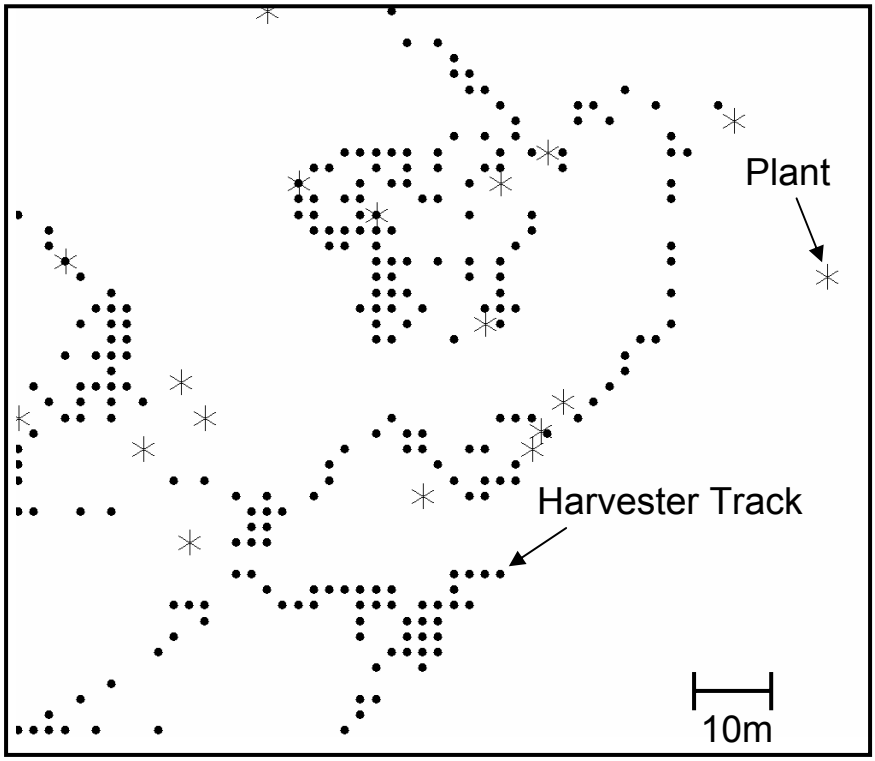


Figure 2.3: Predicted values of stage specifc fitness ($SIF_i$) for adult and juvenile plants in each harvest simulation (1-4) under three harvest regimes (no harvest, harvest, harvest taking seeds)

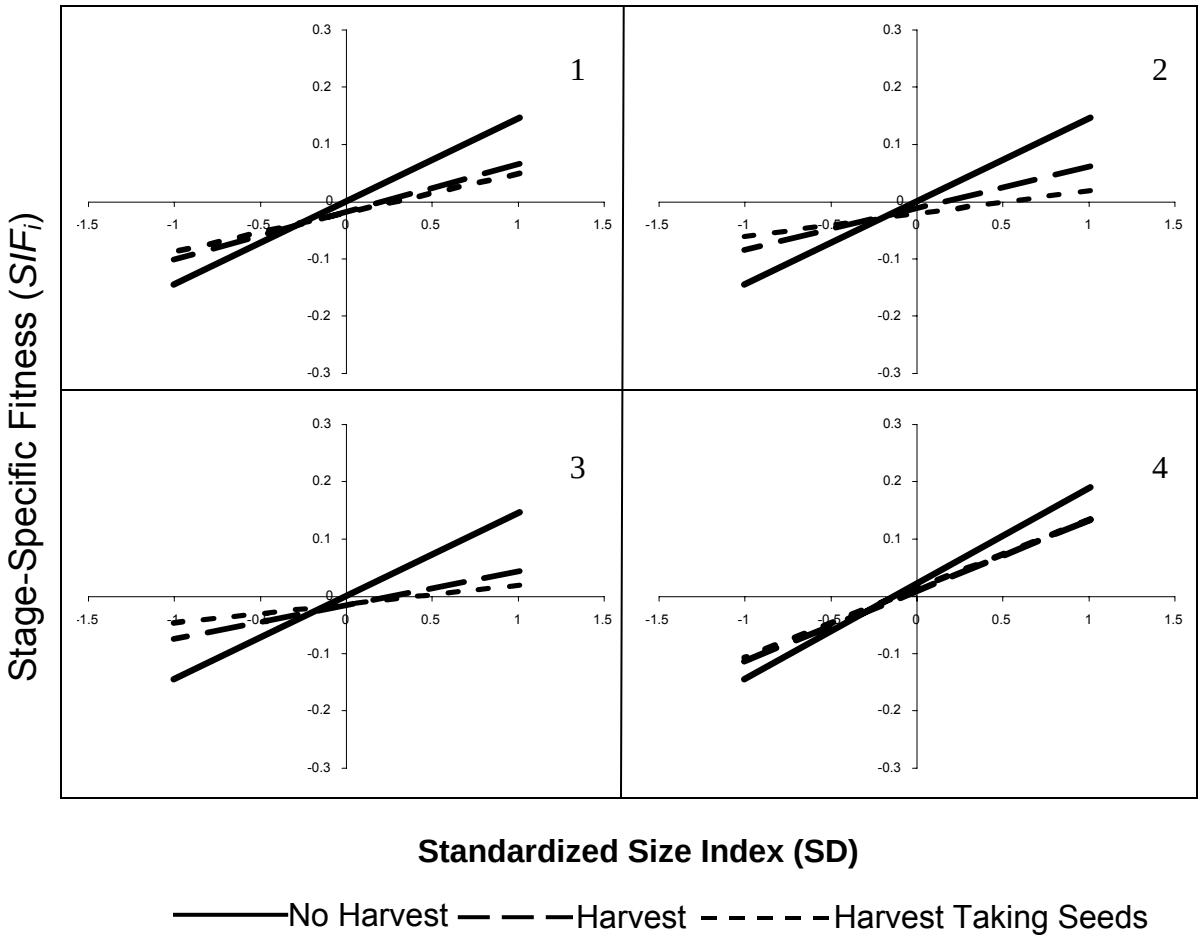
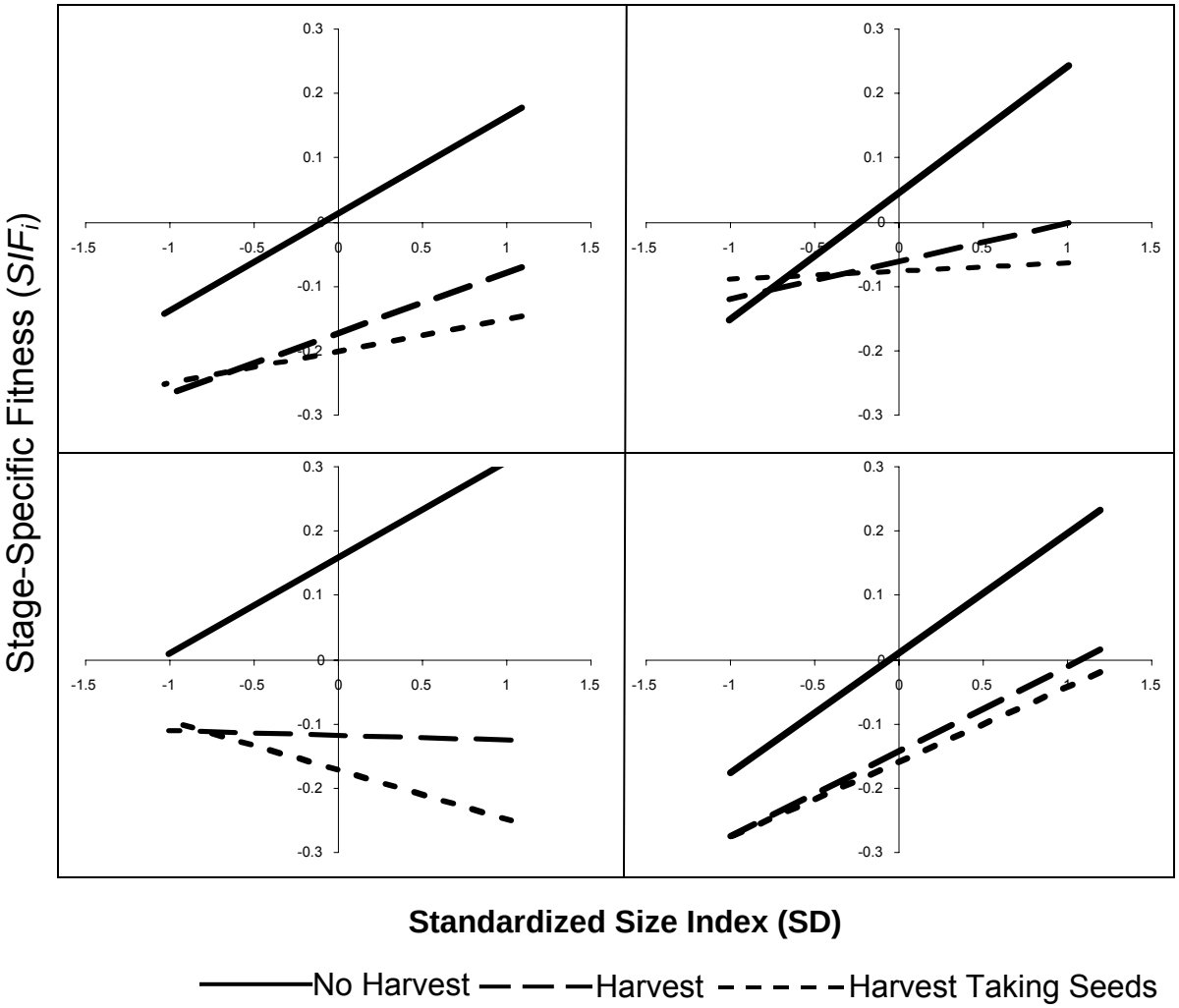


Figure 2.4: Predicted values of stage specifc fitness ($SIF_i$) for adult plants encountered in each harvest simulation (1-4) under three harvest regimes (no harvest, harvest, harvest taking seeds)



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# CHAPTER 3

## Harvest pressure leads to life history changes in American ginseng, *Panax quinquefolius* L. (Araliaceae)[2]

[2]This chapter is formatted for submission to the journal *Biological Conservation*

**Abstract**

As seen in exploited animal species, size-selective harvest occurs in *Panax quinquefolius* L. (American ginseng), a perennial plant of the eastern United States. As harvest is fatal, there is potential for evolutionary change if selected traits are genetically based. In this study, we compared traits potentially affected by selective harvest among 12 populations with different harvest pressures. We used the demographic recovery pattern of an experimentally-harvested population to develop a harvest index: the proportion of seedlings and juvenile plants. Age (estimated from rhizome scars) was related to leaf area, sympodium height and reproduction of plants in populations across a range of harvest indexes. The relationship of size and reproduction to age varied among populations with different harvest indexes in two of three study years; a plant in a population with a high harvest index had reduced sympodium height and an increased likelihood of setting fruit than a plant of the same age in a population with a low harvest index. We also assessed heritable variation in a separate common environment study: leaf area differences were maintained among plants from eight populations four to five years after transplantation. Our results suggest that early fruit set has resulted from fitness advantages conferred by this trait in harvested populations, while reduced sympodium height may be the result of apparency-mediated selection.

## Introduction

Rapid evolution in response to anthropogenic environmental change has been documented in a wide variety of wild taxa (Hendry and Kinnison, 1999; Bone and Farres, 2001; Palumbi, 2001). Classic examples include industrial melanism in moths (*Biston betularia*) (Majerus et al., 2001) and heavy-metal tolerance in plants (Antonovics and Bradshaw, 1970). Humans also directly affect wild species through harvest, which can have dramatic evolutionary effects if it leads to individual mortality. Harvested populations can show reduced diversity if genotypes are lost or novel trait evolution if selective harvest occurs on the basis of heritable traits (Ashley et al., 2003). Evidence for evolutionary change as a result of selective harvest is most notable from commercial fisheries (Law, 2000; Conover and Munch, 2002; Conover et al., 2004). In this context, harvest is selective such that large fish are preferentially taken because they are more valuable or because management efforts often focus on protecting juvenile individuals to sustain fisheries (Law, 2000). Often, these changes occur in directions that are not favorable to human interests, for example reduced growth rate and size at sexual maturity (Conover and Munch, 2002; Stockwell et al., 2003; Coltman et al. 2003). These changes can contribute to the collapse of fisheries and decline of fish stocks even after commercial fishing has been prohibited (Law, 2000). Beyond fisheries, trophy hunting has also led to declines in body mass and horn size in a population of exploited bighorn rams (Coltman et al., 2003) and perhaps also to the evolution of tusklessness in African elephants (Jachmann et al., 1995). The strength of anthropogenic selection and the possibility for rapid evolution in long-lived species have made these issues additional conservation concerns for harvested species (Palumbi, 2001; Ashley et al., 2003).

Animal species provide dramatic examples of the unintended evolutionary consequences of harvest, which can have far-reaching social and economic impacts. However, the number of exploited animal species is dwarfed by the numbers of wild plant species targeted by timber harvest, medicinal use, the ornamental trade and as wild foods (Lebig, 1992; Salick, 1995; Hamilton, 2004, Ticktin, 2004). Given the scope of wild-plant harvest, it is then expected that evolutionary effects have been documented similar to those noted from animal species. The majority of these studies in plants have focused on timber species, probably because of their worldwide economic importance (Ledig, 1992; Buchert et al. 1997; Rajora et al. 2000; Jennings et al. 2001; Sokol et al., 2004; Cornelius et al., 2005; Cloutier et al. 2006). Timber harvest has been generally connected with declines in genetic diversity (but see Cloutier et al., 2005), and the practice of selective logging or high-grading seems to subject tree species to pressures analogous to those of fisheries (Jennings et al., 2001; Sokol et al., 2004; Cornelius et al., 2005).

In addition to fiber and timber, it is estimated that 10-18 percent of the world's flora, or roughly 53,000 species, are used in traditional medicine or western medicine (Hamilton, 2004). For this economically and culturally significant class, the evolutionary impacts of wild harvest have been investigated for American ginseng *Panax quinquefolius* L. This native forest plant is the focus of the largest international trade in medicinal plants from North America (Carlson, 1986), which warranted *P. quinquefolius*' listing in Appendix II of the Convention on International Trade in Endangered Species

(CITES) in 1973 (Robbins, 2000). Although *P. quinquefolius* is cultivated, wild roots are ten times more valuable because they are considered more potent in Asian medicine (Robbins, 2000; USFWS, 2006). Harvest of the root is fatal to the plant, which has led authors to study how this could impact genetic diversity within populations (Cruse-Sanders and Hamrick, 2004a; Cruse-Sanders et al., 2005). Simulated removal of just 10 to 30 percent of plants resulted in significant decreases in genetic diversity (Cruse-Sanders et al., 2005). In addition, extant populations in protected areas had significantly greater diversity than populations in areas open for harvest (Cruse-Sanders and Hamrick, 2004a). As in other exploited species, harvest of *P. quinquefolius* is also size selective (Mooney and McGraw, 2007), which occurs for several reasons. First, harvest is limited by law to plants at least 5-years old and/or those with at least 3 leaves in most of the U.S. states (USFWS, 2006). Second, although harvester behavior is notably difficult to ascertain (Van der Voort and McGraw, 2006), harvesters are also likely motivated to leave behind juvenile plants. Larger plants yield more valuable roots and there is a traditional conservation ethic among some harvesters that motivate them to leave behind smaller plants (Price, 1960; Van der Voort and McGraw, 2006). Third, larger plants are more apparent to human harvesters in the dense understory where *P. quinquefolius* grows, as documented by an experimental study (Mooney and McGraw, 2007).

For size-selective harvest to cause evolutionary changes in *P. quinquefolius* similar to those observed in animal species, the selected traits would need to be genetically based. Most methods to determine the proportion of phenotypic variation that is genetically based (i.e. broad-sense heritability) rely on growing offspring of controlled

crosses to maturity (Falconer and Mackay, 1996). Because as many as eight years may be required between generations, we sought an alternative to assess if age-size relationships have a genetic basis for *P. quinquefolius*. Common garden experiments are a classic technique to determine if differences among populations are genetically based (Turresson, 1922; Clausen et al., 1939). If carryover effects from the original environment are minimized, differences among populations that persist in the common garden are attributable to genetic differentiation (Clausen et al., 1939). We applied this approach to *P. quinquefolius* to assess the degree to which size-related traits were genetically based.

The purpose of this study was to determine if populations of *P. quinquefolius* subject to different harvest histories vary in traits potentially affected by size-selective harvest. This required us to reconstruct the harvest history of our study populations. We developed an index of harvest history based on the results from an experimentally harvested population (Van der Voort et al., 2003) and its recovery 10 years post-harvest. Given that *P. quinquefolius* exhibits a stage-dependent life-cycle, selection on size has the potential to affect life-history traits. To assess variation in life-history, plants in the study populations were aged by counting scars along the rhizome; this allowed us to compare how growth rate and the timing of reproduction varied among populations with different harvest histories.

## Methods

**Study species.** American ginseng, *Panax quinquefolius* L. (Araliaceae) is a perennial

herb infrequently found in the understory of the eastern deciduous forest in the United States (Anderson et al., 1993; McGraw et al., 2003). Although widespread in its distribution, it can be locally rare due to nearly three centuries of commercial harvest, habitat degradation and increased browse by white-tailed deer (McGraw et al., 2003; McGraw and Furedi, 2005). The life-cycle of *P. quinquefolius* is structured into stage classes based upon the number of leaves. Seedlings consist of a single compound leaf for the first few (2-5) years of growth. Juvenile plants have two compound leaves and adult plants typically have three compound leaves, with plants possessing more leaves being less common in the wild (Lewis and Zenger, 1982; Charron and Gagnon, 1991 McGraw and Furedi, 2005). Plants frequently initiate flowering as juveniles, but these juveniles produce seeds only infrequently (Carpenter and Cottam, 1982). Most adult plants are reproductive, producing an umbel of small flowers at anthesis in midsummer (Carpenter and Cottam, 1982; Schlessman, 1985). Flowers of *P. quinquefolius* are self-compatible, but are also visited by generalist pollinators (Carpenter and Cottam, 1982). Seeds are borne in fleshy berries that turn red upon ripening in late summer (McGraw et al., 2005). Seeds exhibit variable dormancy periods, but most seeds germinate in the second spring following dispersal (McGraw et al., 2005).

**Study populations.** Twelve study populations were located in six states (IN, MD, NY, PA, VA and WV) central to the range of *P. quinquefolius*; each population was given a two letter acronym to protect the details of its location (Table 3.1). In the smallest population, we observed an average of 46 plants in each study year while the largest population contained an average of 349 plants in total. Populations were located on both

public and private lands through a combination of field surveys and consultations with harvesters and land managers. When the populations were initially located, the areas were intensively searched to find all individuals. The populations were marked and mapped following the techniques of McGraw and Furedi (2005): plants were marked with a buried aluminum nail engraved with a unique number and their locations were then mapped using a "Phototrail" system, in which distance and angle measurements led us to each plant or cluster through a series of digital photographs.

**Population harvest index.** In one previous study of genetic diversity differences, populations were classified as either unprotected or protected based on their location in areas where harvest is permitted or forbidden, respectively (Cruse-Sanders and Hamrick, 2004a). However, most laws guiding harvest are nearly unenforceable in practice (Robbins, 2000) and poaching from protected areas may be commonplace (Van Manen et al., 2005). Therefore, the protection status of a population would be at best an approximate indicator of harvest history. To develop an index of harvest pressure, we used data from an experimental harvest of a naturally-occurring *P. quinquefolius* population (Van der Voort et al., 2003). All individuals regardless of size were removed in 1996 and the area was censused from 1997 to 2001 (Van der Voort et al., 2003). In the first five years following harvest, the population was dominated by 1-leaved seedlings and juvenile plants (Van der Voort et al., 2003); therefore, we continued to census the population from 2002 to 2006 to track recovery at 10 years post-harvest. Based on this recovery pattern, we used the proportion of all plants that were 1-leaved seedlings and 2-leaved juvenile plants as a harvest index in the study 12 populations.

**Measuring size and age of plants.** From 2004 to 2006, the 12 populations were visited twice yearly to gather demographic data. In late May through early June, individual plants were measured and the populations were searched for new seedlings, which are identifiable by an attached seed coat. Several measurements were taken on all plants: sympodium height, length and width of longest leaflet of all leaves and reproductive status (determined by the presence of an immature inflorescence). The leaflet length (LL) and width (LW) were used to estimate the area of each compound leaf using the equation:

$$LA = 11.4597 + 4.5774LL - 4.5091LW + 0.5786LL \times LW .$$

This equation was found by regressing the leaf areas measured from 101 adults (5 leaflets per leaf) and 59 seedlings (3 leaflets per leaf) on the model containing leaflet length and width terms that yielded the best fit ($r^2 = 0.96$). For juvenile and adult plants, total leaf area was then found by adding each separate estimated leaf area. Populations were revisited in August to record fruit set and the number of seed produced. Fruits of *P. quinquefolius* are typically either 1 or 2-seeded in the wild, which can be determined without dissection of fruits. Plants that were browsed by white-tailed deer were identifiable by partial or complete loss of aboveground parts (Furedi and McGraw, 2004).

In *P. quinquefolius*, scars left by the annual abscission of the sympodium along the subterranean rhizome provide an estimate of age; this technique has been used previously in *P. quinquefolius*, and it is in fact the standard set out by the USFWS to guide harvesters to comply with age restrictions (Carpenter and Cottam, 1982; Lewis and

Zenger, 1982; Anderson et al., 1993; USFWS, 2006). In July of 2005 and August of 2006, randomly-selected subsets of plants in each population were aged by counting annual abscission scars. Beginning at the base of the sympodium, the soil was carefully excavated from around the rhizome. Plants only rarely produce two sympodia in a given year (Lewis and Zenger, 1982), so each scar along the rhizome was considered to be one year of growth. New seedlings were considered to be age zero at their appearance in the population and one year old if they persisted to the following census year.

**Data analysis.** We used multiple regression analysis to determine if the way size traits change with age (i.e., growth rate) varies between populations with different harvest histories (Sokal and Rohlf, 1995). Significance of the model term 'Age X Harvest Index' would indicate that the size-age relationship varied among populations with different harvest indexes. We also examined how reproduction varied with age. For juvenile and adults plants, we used a logistic regression to determine how age predicts whether a plant is classified as reproductive, i.e., produces an inflorescence (Sokal and Rohlf, 1995). Among those plants classified as reproductive, we again used a log-likelihood test to determine if the influence of age on the probability of setting fruit varied with harvest index. Plants that were browsed by deer or otherwise missing prior to data collection for seeds were excluded from these analyses. For the subset of plants that set fruit, we in turn examined if the number of seeds produced by a plant of a given age varied among populations with different harvest indexes using multiple regression. All continuous dependent variables (leaf area, sympodium height and number of seeds) were natural-log transformed to improve the normality of residuals. To evaluate one potential

environmental factor that could confound harvest index and plant growth, we compared browse rates among populations with different harvest indexes using a logistic regression. Browse rates were calculated as the average proportion of plants that were browsed across the three study years. We performed all analyses using SAS JMP v 6.0 (SAS Institute Cary, 2005).

**Common garden.** In 2002 and 2003, a living germplasm bank for *P. quinquefolius* was created at a protected site in New York State from plants collected from wild populations in eight states (KY, MD, ME, NC, NY, OH, TN and VA) (Beyfuss, personal comm.). Because it was intended as germplasm bank rather than a common garden, the roots were planted non-randomly (in rows) and the plants were all mostly reproductive adults. However, the germplasm bank does minimize carryover effects from the plants' original environments: transplants were as bare roots and they had likely acclimated to the site. In June of 2006, we measured sympodium height and leaflet size on the plants, and we also aged plants at this time using the technique described previously. Because the plot was small (5 X 10 m), we considered the plot a 'common environment' such that each individual was considered randomly placed for statistical purposes. We tested for differences among populations in sympodium height and leaf area with analysis of covariance, where age was used as a continuous covariate.

## Results

**Experimental harvest.** As of 10 years post-harvest, the experimentally-harvested population had not recovered its stage distribution, which was originally dominated by

reproductive adults (Fig. 3.1). The population consisted of 21.7% seedlings and juvenile plants prior to harvest in 1996, but in 2006 62.5% of the population remained in these stages.

**Harvest index and age.** For the 12 study populations, harvest index ranged between 0.41218 for the VC population and 0.95285 for the AD population (Table 3.1). The variation in this value indicates that the populations represent a range of harvest pressures. In addition, two incidences of harvest took place following the August census of 2004 and prior to the May census of 2005: 31% of plants were harvested from the AD population and 14% of the plants were harvested from the CC population. Similarly, following the August census of 2005 but prior to May of 2006, 18% of plants were harvested from the EB population.

Altogether, we collected age data for 683 total plants among the 12 study populations. Relatively few plants were observed that were greater than 20 years of age (n = 23), and these occurred entirely in populations with lower harvest indices (Fig. 3.2). To ensure that the relationship of age to size and reproduction would be evenly characterized across harvest index, we excluded plants greater than 20 years of age from the subsequent analyses. The average proportion of plants that were browsed by white-tailed deer did not differ among populations with different harvest indexes (n=583; $\chi^2$ = 1.1000, p = 0.2948)

**Leaf area.** Slightly different numbers of plants were used in the analyses among years (2004, 2005, 2006), as some plants were browsed prior to data collection and more plants were present in most populations as the census period continued (Supplementary data, Table 3.2). Age was a positive predictor of leaf area for plants across all populations in all years of study (2004: $b$ = 1.1272; 2005: $b$ =1.1431; 2006: $b$ = 1.1400). In 2004, plants in populations with higher harvest indices has significantly smaller leaf areas ($b$ = -1.4062). In 2006, there was a trend for leaf area to increase slightly in populations with higher harvest indexes. The relationship between age and leaf area did not vary significantly with harvest index in any year of the study. The variation suggested by the trend observed in 2006 was very slight: the predicted leaf area for a 10 year old plant was 87.9 $cm^2$ in a population with a low harvest index (HI = 0.4) compared to 89.2 $cm^2$ in a population with a high harvest index (HI = 0.9).

**Sympodium height.** Again, slightly different numbers of plants were included in the analyses for sympodium height across the study years (Supplementary data, Table 3.3). In all years, sympodium height increased significantly with age, as expected (2004: $b$ = 1.0361; 2005: $b$ = 1.0493; 2006: $b$ = 1.1065). In 2004 and 2005, sympodium height differed significantly with harvest index (2004: $b$ = -1.2942; 2005 $b$ = -1.4742); both indicated that sympodium heights were smaller in populations with high harvest indices. In 2005 and 2006, the age-sympodium height relationship varied significantly with harvest index. Sympodium height increased more with age in populations with lower harvest indexes (Fig. 3.2b). For example, a 10 year old plant would be 14.1cm in height

in a low harvest index population (HI = 0.4) whereas a plant of the same age would be 10.6cm in height in a high harvest index population (HI = 0 .9).

**Reproduction**. Nearly all (95.8%) adult plants were classified as reproductive, but flowering was more variable among juvenile plants with a mean of 55.2% juveniles classified as reproductive in 2004, 2005 and 2006. Age significantly influenced the likelihood of a plant producing an inflorescence in every year of study (Supplementary data, Table 3.4). The probability of flowering consistently increased with age (2004: $b$ = 0.0989; 2005: $b$ = 0.2491; 2006: $b$ = 0.2550). The likelihood of flowering did not vary significantly with harvest index in any year, but there was a trend for fewer juvenile and adult plants to be classified as reproductive in 2004 ($b$ = -4.2178). Harvest index did not alter the age-flowering relationship in any year of study.

Unlike flowering, age did not consistently predict the likelihood that a flowering plant would set fruit (Supplementary data, Table 3.5). The probability of fruit set increased with age in 2004 and 2005 ($b$ = 0.1012 and $b$ = 0.1063, respectively), but this relationship was not significant in 2006. Harvest index did not influence the probability that a reproductive plant would set fruit in 2004 and 2005, but in 2006 the probability of fruit set decreased as harvest index increased ($b$ = -4.7193). Harvest index of a population significantly altered the age-fruit set relationship of reproductive plants in 2004 and 2005. The probability that a plant would set fruit increased more with age in populations with high harvest indexes relative to population with low harvest indexes

(Fig. 3.4). However, there was a trend in 2006 for fruit set to decrease in populations with higher harvest indexes.

Age was not a consistent predictor of the number of seeds for reproductive plants that set fruit (Supplementary data, Table 3.6); only in 2004 was there a trend that suggested the number of seeds increased with age ($b$ = 1.2209). In both 2005 and 2006, the numbers of seeds produced was significantly less in populations with higher harvest indexes (2005: $b$ = -3.1557, 2006: b = -2.4636). The relationship of age to number of seeds produced by reproductive plants did not vary with harvest index in any year of study.

**Common garden.** From the eight populations, 29 transplanted individuals emerged in 2006. When age was used as a continuous covariate, there were significant differences in leaf area among the populations from ($F = 3.1263$, $p = 0.0213$). We also found a trend for sympodium height to vary among plants originating from the eight different populations ($F = 2.882$, $p = 0.0694$).

## Discussion

Harvest events can have profound effects for targeted species, but they do not necessarily extirpate populations. For *P. quinquefolius,* the distribution of individuals among stage classes remained skewed towards seedlings and juvenile plants 5 year after the experimental harvest (Van der Voort et al. 2003). As of 2006 (ten years post-harvest), the population continued to be dominated by seedlings and juvenile plants. The

fact that stage structure has not recovered to pre-harvest levels is likely due to the intensity of the experimental harvest, i.e., all aboveground plants were removed (Van der Voort et al., 2003). Although one juvenile plant 'escaped' harvest by having no aboveground portion in 1996 (Van der Voort et al., 2003), all other plants are presumably the result of recruitment from the soil seed bank. Had the experimental harvest left behind more plants, we would expect recovery of stage structure to occur more rapidly. Thus, in following the recovery of the harvested population, we have mostly tracked the growth of individuals from seeds. Some of the seedlings that emerged in the first spring following harvest have reached adult size by 2006, but later germination and the duration of the juvenile stage continues to skew the stage distribution. We would likely see the original stage distribution return given a longer recovery period and no additional harvest events.

Based on the recovery pattern observed from the experimental harvest, the proportion of seedlings and juveniles provides an index of population harvest pressure. Results from aging nearly 700 plants also reveal the 'fingerprint' of a mortality event in populations with high harvest indexes: no plants 20 years of age or greater were in the four populations with harvest indexes greater than 0.75. Alternative explanations that would yield this impact on age structure *and* higher numbers of seedlings and juvenile plants could be developed, for example recent colonization or environmental disturbance. However, this harvest index is supported by results reported by a previous study: 13 populations in unprotected (likely harvested) areas were composed of significantly more

seedlings and juveniles than observed in 8 protected populations (Cruse-Sanders and Hamrick, 2004).

By examining the age-size relationship in populations across a range of harvest histories, we have tested for potentially harvest-induced evolutionary change. In terms of leaf area, there were no differences in the size-age relationship with respect to harvest index. However, plants in populations with higher harvest indices had reduced sympodium heights in two of three study years. Furthermore, the relationship of sympodium height to age varied among populations with different harvest indexes in two of three years. In other words, plants in populations with high harvest indexes had both small sympodium heights and slow growth rates for this trait relative to populations with low harvest indices. In a related study, *P. quinquefolius* plants with greater leaf areas and sympodium heights had reduced fitness when their population experienced harvest (Mooney and McGraw, 2007). Notably, this fitness difference occurred because large adult plants were simply more apparent to the human harvesters (Mooney and McGraw, 2007). Sympodium height is likely an important component of apparency in the dense understory where *P. quinquefolius* grows. The reduced sympodium heights are similar to results from herbarium specimens of *P. quinquefolius* collected from 1900 to 2001 (McGraw, 2001). Of the 11 measured traits, sympodium height along with peduncle height showed the largest declines, 25.8% and 42.7% respectively (McGraw, 2001). These declines were circumstantially linked to harvest as they were most pronounced in specimens from regions of intensive harvest. Simultaneous environmental factors could not be discounted (McGraw, 2001); specifically, following browse by white-tailed deer

plants often regress to a smaller stage class in the following year (McGraw and Furedi, 2005). However, the browse rates we observed did not vary with harvest history index in our 12 study populations.

For the observed changes in size as a result of selective harvest to be possible, a significant portion of the observed phenotypic variation would need to be genetically based. From the common garden study, we observed that plants from different states maintained different leaf areas and perhaps sympodium heights 3-4 years after transplantation. For this observed variation among populations to be attributable to genetic differentiation, carryover effects from their original environment would need to be minimal. When the common garden technique is used on plants rather than seeds, researchers assume that carryover effects in transplants are reduced over time (Gallagher et al., 1988; Pors and Werner, 1989; Thompson et al., 1991). Given that plants have been at the site multiple years, we would expect carryover effects to be minimal, despite whole roots being initially transplanted. Furthermore, using age as a covariate allowed us to account for its influence on plant size. Altogether, results from the common garden indicate some heritability for size traits in *P. quinquefolius*.

From the reproductive results, we also see changes that may be brought about by harvest. The probability that a flowering plant of a given age would set fruit was mediated by harvest index in two of three census years. As measured, a plant setting fruit means it produced at least on seed in that year. In this way, plants from high harvest index populations were more likely to have produced at least one seed than plants of the

same age in low harvest index populations. Similar changes have been observed in fish species, where exploited stocks achieved reproductive maturity at earlier ages and smaller sizes (Law, 2000). These changes can be brought about as size-selective harvest will simultaneously be age-selective if larger individuals tend to be older. In *P. quinquefolius* for example, if the onset of reproduction occurs at seven years of age for most plants, and harvest targets plants that are eight years of age or older, plants producing any seeds before seven years would leave marginally more offspring. The presence of a long-lived seed bank and its importance in a population's recovery from harvest may reinforce this fitness advantage. As seen in other plant species, soil seed banks can be influential mediators to evolutionary change (Levin, 1990). If a earlier reproduction is conferring fitness 'benefits' in frequently harvested populations, it would follow from life history theory that this change would occur at some 'cost' (Stearns, 1989). From our results, numbers of seeds produced was significantly less in populations with high harvest indexes in two of the three study years. If plants are setting fruit at earlier ages in populations with high harvest indexes, these individuals may lack sufficient carbon reserves to produce many fruits. Similar results have been documented in many teleost fish species subject to size-selective harvest: earlier reproduction is favored although younger females produce smaller eggs (Rochet et al. 2000). Alternatively, reduced numbers of seeds could be the result of pollen limitation from fewer reproductive plants in harvested populations. This Allée effect was demonstrated in *P. quinquefolius* by an experimental study whereby increased seed production occurred at higher plant densities (Hackney and McGraw, 2001).

As the change in age-at-reproductive maturity we observed mirrors that of exploited fish stocks, we must also address similar concerns about the interpretation of results. Specifically, we must take into consideration the simultaneous reduction in densities of conspecifics that occurs with harvest (Law, 2000; Conover and Munch, 2002; Conover et al., 2004). Reduction in intraspecific competition can increase food availability such that earlier reproduction can occur independently from the effects of selection (Law, 2000; Conover and Munch, 2002; Conover et al., 2004). The extent of competition in *P. quinquefolius* populations in unknown, but it is likely minimal for several reasons. For one, densities of *P. quinquefolius* plants in wild populations are characteristically low: surveys of forested sites yielded a density of 14.7 plants per hectare on private land versus 2.6 plants per hectare on public land (McGraw et al., 2003). Although these densities seemingly place plants at distances where competition would be minimal, plants are typically clumped within populations (Cruse-Sanders and Hamrick, 2004b). But slow-growing understory plants like *P. quinquefolius* generally show extended longevity of tissues and modest mineral nutrient demands, which could confer tolerance to resource competitions (Reich et al. 2003). Also as in fisheries, harvest-induced evolutionary change may be complicated by simultaneous environmental variation (Rijnsdorp, 1993). Environmental quality and harvest index could be potentially correlated if harvesters are preferentially targeting worse sites for *P. quinquefolius*. It is difficult to reconcile how release from competition by harvest or low quality environments could simultaneously lead to reduced sympodium growth and earlier reproductive maturity in terms of fruit set in *P. quinquefolius*.

Combined with the previous experimental demonstration of size-selection by harvesters (Mooney and McGraw, 2007), our results indicate that harvest pressures can cause evolutionary change in *P. quinquefolius*. One far-reaching effect in *P. quinquefolius* would occur if biomass allocation to roots is sacrificed by reduced sympodium heights or earlier fruit set. Indeed, the numbers of roots per kilogram has increased (i.e., roots are smaller) since these data have been collected by the USFWS from states in compliance with CITES (USFWS, 2006). Reductions in root sizes would affect harvesters directly: they may need to collect more roots to achieve reasonable economic returns or harvesting could no longer be worth their efforts. Similar diminishing returns have also been characteristic of overharvested fisheries, where decreases in age-at-maturity also result in smaller fish (Law, 2000).

The scope of wild-harvest worldwide suggests that our results may have parallels in other species (Hamilton, 2004). Although slower growth of trees left-behind by selective timber harvest has been documented (Sokol et al., 2004) analogous results for herbaceous plant species are less common. In a study of the *Saussurea laniceps* (Himalayan snow lotus), the authors found smaller flowering individuals in harvested areas compared to protected areas (Law and Salick, 2005); because age was not included in their analyses, individuals could be flowering at smaller sizes or harvested populations may simply consist of younger plants. Weeding pressure is also analogous to selection through harvest, although the intention from the human perspective is different. Life history changes have been brought about by weeding; for example in *Senecio vulgaris*, plants in well kept gardens developed faster than plants from unmanaged sites (Kadereit

and Briggs, 1985; Briggs et al., 1992). The putative fitness advantages of an accelerated life-cycle were twofold: earlier flowering reduced size and apparency, and it increased the probability that an individual will produce seeds prior to being weeded out (Briggs et al., 1992). Remarkably, we see similar reductions in growth accompanied by earlier reproduction in *P. quinquefolius*.

Overall, our results extend observations from animal species to plants, which represent a significant portion of the worldwide biodiversity used by humans. Evidence for selective harvest in other plant species would indicate that the effects we observed may be more widespread. For example, specimens of *Dioanaea muscipula* (Venus' fly trap) confiscated from harvesters had significantly larger petioles and traps than plants left behind (Luken et al., 2005). Although exploitation typically ranks behind habitat destruction and invasive species as a threat to native plant species (Wilcove et al., 1998), we see how wild-harvest can have subtle, far-reaching impacts. The life history changes we observed in *P. quinquefolius* confirm that humans can have dramatic evolutionary impacts in wild populations, even for long-lived, slow growing species.

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Table 3.1: Study populations, their locations, population sizes averaged from 2004-2006, and harvest indexes. Harvest index of the populations was estimated from the proportion of 1-leaf and juvenile plants.

| Population | Location | *N* | Harvest Index |
|---|---|---|---|
| VC | Vermillion Co, IN | 173 | 0.41218 |
| EP | Lancaster Co, PA | 99 | 0.41727 |
| EB | Preston Co, WV | 46 | 0.50574 |
| HP | Albany Co, NY | 280 | 0.52864 |
| CC | Garrett Co, MD | 154 | 0.65384 |
| LK | Franklin Co, PA | 349 | 0.68791 |
| GB | Greenbrier Co, WV | 123 | 0.72131 |
| TP | Albany Co, NY | 62 | 0.72348 |
| TR | Parke Co, IN | 133 | 0.78020 |
| PO | Bedford Co, VA | 300 | 0.78287 |
| AD | Mercer Co, WV | 75 | 0.84864 |
| RD | Pulaski Co, VA | 129 | 0.95825 |

Table 3.2: Results from regression of leaf area on age and harvest index for three study years.

| *Year* | | *Model Term* | *F Ratio* | *p-value* |
|---|---|---|---|---|
| 2004 | *n* = 454 | Age | 377.6508 | <0.0001 |
| | | Harvest Index | 4.1641 | 0.0419 |
| | | Age X Harvest Index | 0.0120 | 0.9127 |
| 2005 | *n* = 537 | Age | 811.6321 | <0.0001 |
| | | Harvest Index | 0.4813 | 0.4881 |
| | | Age X Harvest Index | 0.0779 | 0.7803 |
| 2006 | *n* = 656 | Age | 11118.7000 | <0.0001 |
| | | Harvest Index | 3.7275 | 0.0540 |
| | | Age X Harvest Index | 3.6721 | 0.0558 |

Table 3.3: Results from regression of stem height (ln transformed) on age and harvest index for three study years.

| *Year* | | *Model Term* | *F Ratio* | *p-value* |
|---|---|---|---|---|
| 2004 | *n* = 454 | Age | 91.0958 | <0.0001 |
| | | Harvest Index | 5.7398 | 0.0170 |
| | | Age X Harvest Index | 0.3250 | 0.5689 |
| 2005 | *n* = 537 | Age | 202.0939 | <0.0001 |
| | | Harvest Index | 13.2837 | 0.0003 |
| | | Age X Harvest Index | 5.7995 | 0.0164 |
| 2006 | *n* = 659 | Age | 350.7916 | <0.0001 |
| | | Harvest Index | 0.1327 | 0.7157 |
| | | Age X Harvest Index | 9.6198 | 0.0020 |

Table 3.4: Results from logistic regression of reproductive status (presence/absence of inflorescence) on age and harvest index for juvenile and adult plants observed for three study years.

| *Year* | | *Model Term* | *Likelihood-Ratio* $\chi^2$ | *p-value* |
|---|---|---|---|---|
| 2004 | *n* = 411 | Age | 11.9852 | 0.0005 |
| | | Harvest Index | 3.2586 | 0.0710 |
| | | Age X Harvest Index | 0.2712 | 0.6025 |
| 2005 | *n* = 405 | Age | 44.9529 | <0.0001 |
| | | Harvest Index | 2.5819 | 0.1081 |
| | | Age X Harvest Index | 1.9120 | 0.1667 |
| 2006 | *n* = 374 | Age | 50.2933 | <0.0001 |
| | | Harvest Index | 0.0780 | 0.7800 |
| | | Age X Harvest Index | 0.9164 | 0.3384 |

Table 3.5: Results from logistic regression of fruit set (yes/no) on age and harvest index for reproductive plants over three study years; observations exclude plants that were browsed or otherwise missing prior to collection of data in August of each year.

| *Year* | | *Model Term* | *Likelihood-Ratio* $\chi^2$ | *p-value* |
|---|---|---|---|---|
| 2004 | *n* = 207 | Age | 6.9056 | 0.0086 |
| | | Harvest Index | 2.5977 | 0.1070 |
| | | Age X Harvest Index | 4.10167 | 0.0451 |
| 2005 | *n* = 290 | Age | 11.3112 | 0.0008 |
| | | Harvest Index | 0.1724 | 0.6780 |
| | | Age X Harvest Index | 4.8754 | 0.0272 |
| 2006 | *n* = 234 | Age | 1.4890 | 0.2224 |
| | | Harvest Index | 27.8010 | <0.0001 |
| | | Age X Harvest Index | 3.0348 | 0.0815 |

Table 3.6: Results from regression of number of seeds (ln transformed) on age and harvest index for those reproductive plants that produced any seeds in each study year.

| *Year* | | *Model Term* | *F Ratio* | *p-value* |
|---|---|---|---|---|
| 2004 | *n* = 68 | Age | 3.2933 | 0.0742 |
| | | Harvest Index | 2.7137 | 0.1044 |
| | | Age X Harvest Index | 0.0013 | 0.9715 |
| 2005 | *n* = 117 | Age | 2.3832 | 0.1254 |
| | | Harvest Index | 23.0413 | <0.0001 |
| | | Age X Harvest Index | 0.2131 | 0.6452 |
| 2006 | *n* = 108 | Age | 1.7802 | 0.1850 |
| | | Harvest Index | 23.5940 | <0.0001 |
| | | Age X Harvest Index | 0.4541 | 0.5019 |

Figure 3.1: The proportion of seedlings and juvenile plants (harvest index) in an experimentally-harvested population from prior to harvest (1996) to ten years after harvest (2006).

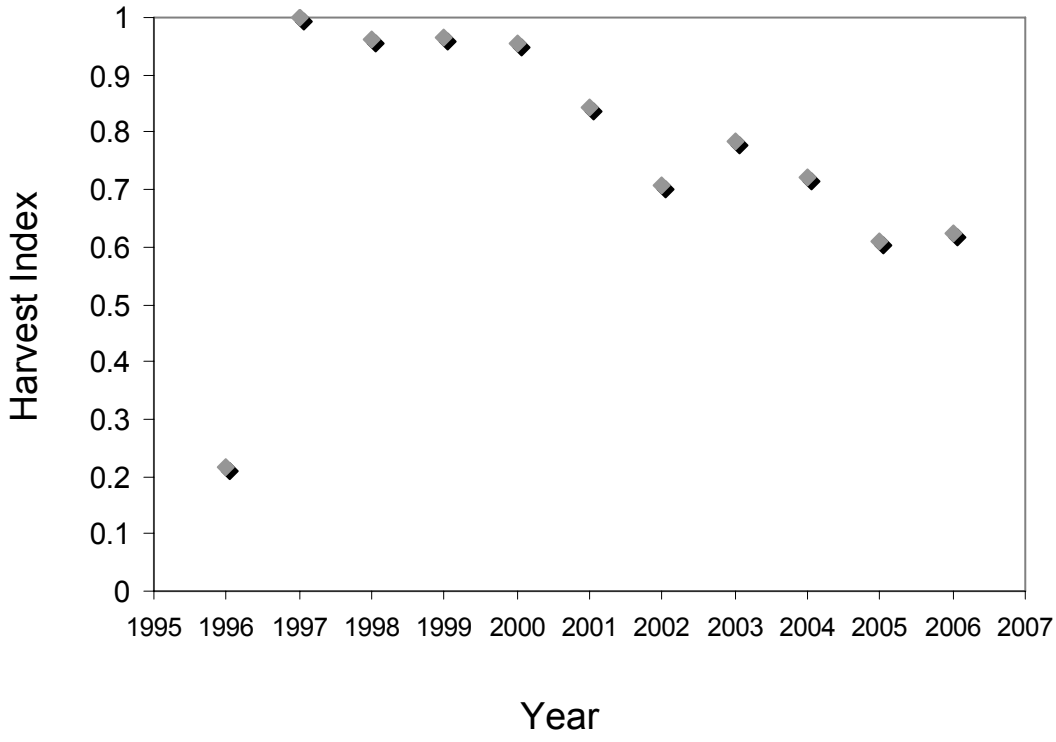


Figure 3.2: Distribution of observations of plant ages among populations with different harvest indexes for 2006.

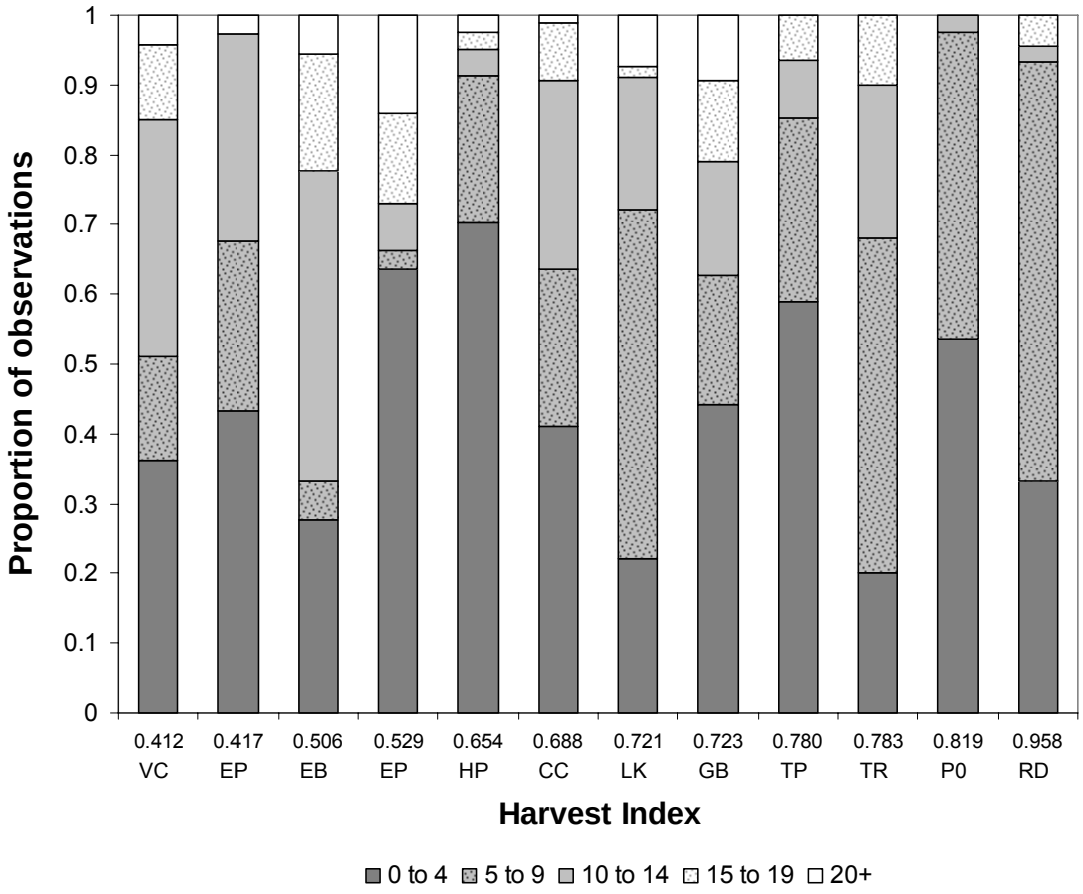


Figure 3.3: The predicted sympodium heights of plants aged 1 to 20 years in populations across the observed range of harvest indexes (0.4 to 0.9). Results depicted are from the regression performed on data collected in 2006.

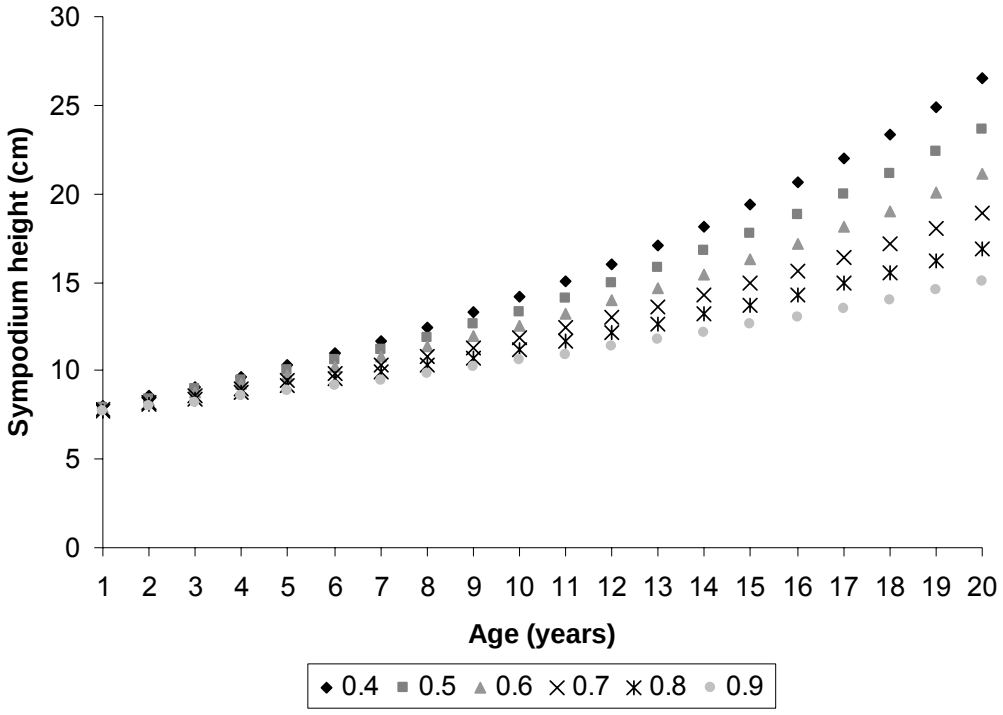
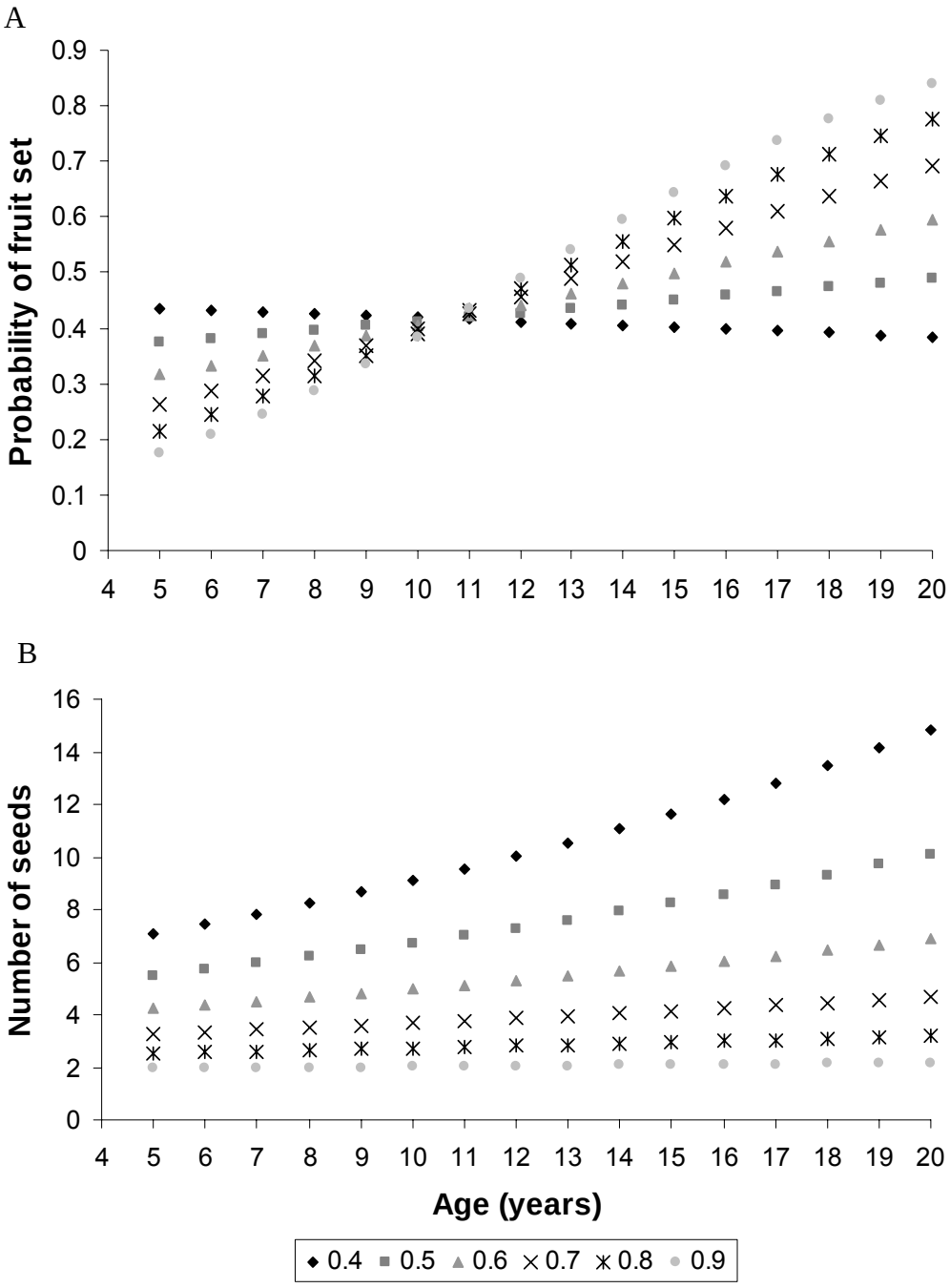


Figure 3.4: The predicted (A) fruit set of reproductive plants and (B) number of seeds in plants that set fruit for plants aged five to 20 years in populations across the observed range of harvest indexes (0.4 to 0.9). Results depicted are from regressions performed on data collected in 2005.



# CHAPTER 4

## Effects of self-pollination and outcrossing with cultivated plants in small natural populations of American ginseng, *Panax quinquefolius* (Araliaceae)[3]

[3] This chapter is formatted for submission to the *American Journal of Botany*: submitted 11/20/06, revised and resubmitted 2/16/07 and is currently in review.

**Abstract**

Fluctuations in plant population sizes and densities can be the direct result of human activity. In small or less dense populations, self-pollination and inbreeding can increase. At the same time, unusual levels of outcrossing can occur through restoration efforts using cultivated source material. To simultaneously study the effects of inbreeding and outcrossing with cultivated genotypes, we performed experimental pollinations using *Panax quinquefolius* L. (American ginseng), a wild-harvested long-lived plant with a mixed mating system. To evaluate inbreeding depression, wild plants were either self-pollinated or cross-pollinated with other plants within the population. Wild plants were also pollinated with pollen from cultivated plants from either West Virginia or Wisconsin to determine fitness consequences of outcrossing at this scale. We also monitored seedlings in eight wild populations to allow us to evaluate how early size characters influence longer-term survival in this long-lived species. In the inbreeding pollination treatments, a significantly higher proportion of self-pollinated flowers produced seeds relative to cross-pollination within the population. The majority of seeds germinated after 20 months of dormancy, and we then monitored the seedlings for survival and measured growth over two years. Offspring of self-pollination showed reduced leaf area and stem height in year two relative to offspring of cross-pollination. For seedlings we monitored in wild populations, leaf area in year two of growth was a significant positive predictor of survival over the following three years. The results from the inbreeding pollination treatments are suggestive of inbreeding depression, which is somewhat unexpected in this highly self-fertile species. The offspring resulting from crosses with Wisconsin cultivated plants showed 127.3% greater leaf area and 164.8%

greater root biomass in year two than the offspring of crosses within the population. The accelerated growth of cultivated offspring indicates genetic differences between wild and cultivated populations. Because outbreeding depression may not be detected in first generation hybrids, the long term fitness consequences of crossing between wild and cultivated genotypes are uncertain.

## Introduction

Given the influence of environmental stochasticity, the determinants of plant reproductive success will likely vary over time as population size and density fluctuate. Plants with mixed mating systems have the ability to respond to such fluctuations. For example, pollinators may be scarce or conspecifics too few to attract pollinators during some flowering periods, in which case self-pollination would prevail. At other times, increased population sizes and densities would provide more opportunities for outcrossing. As with environmental stochasticity, humans can also be agents that affect reproductive success in targeted plant species. For populations reduced in size by human activities, inbreeding depression is an added conservation concern due to the increased likelihood of self-pollination or mating between close relatives in small populations (Ellstrand and Elam, 1993; Crnokrak and Roff, 1999; Hedrick and Kalinowski, 2000; Keller and Waller, 2002). Furthermore, species without an evolutionary history of inbreeding are likely to harbor deleterious recessives (Byers and Waller, 1999). If inbred offspring that express these alleles show reduced fitness, population extinction can be accelerated (Gilpin and Soulé, 1986). At the same time, humans may work to increase population sizes of rare plants through restoration efforts. Striking examples of 'genetic rescue' exist for some plant species, whereby the introduction of outside genotypes has relieved the negative effects of inbreeding depression (Richards, 2000; Newman and Tallmon, 2001; Tallmon et al., 2004). The deliberate introduction of new individuals to populations of rare plants poses some risks, e.g. the introduction of disease and outbreeding depression (Storfer, 1999; Hufford and Mazer, 2003). Specifically, offspring produced by crosses with genetically dissimilar 'rescuers' may show reduced fitness

because of the introduction of novel genes and the breakup of locally adapted gene complexes (Lynch, 1991; Templeton, 1991; Schierup and Christiansen, 1996). Similarly, unintentional gene flow from cultivated plants to their wild relatives (which may be rare or threatened) has received additional attention due to the ecological risks of transgenes (Ellstrand et al. 1999; Lu and Snow, 2005; Chapman and Burke, 2006). Given the potential consequences of reduced population size and well-intentioned management efforts, a rare or threatened plant species can be put risk from both inbreeding depression and outbreeding depression.

To examine these issues in natural populations, we selected *Panax quinquefolius* L. (Araliaceae) American ginseng, a plant for which novel levels of both inbreeding and outcrossing are possible through the direct and indirect effects of human activities. Harvest of the root for export and browse by overabundant deer share the result of reducing local population size in *P. quinquefolius* (Van der Voort et al., 2003; McGraw and Furedi, 2005; Van der Voort and McGraw, 2006), and habitat destruction, invasive species and climate change may also reduce population size in *P. quinquefolius*. These same forces have been responsible for the reduction of population size in many rare plants (Huenekke and Thomson, 1995; Drayton and Primack, 1996; Russell et al., 2001; Ticktin, 2004). Given that an estimated 15 million plants were removed from the wild in 2004 (USFWS, 2005), harvest is a dominant feature in the population biology of *P. quinquefolius*. The scale of harvest from wild populations and export led to the listing of *P. quinquefolius* in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Robbins, 2000). Simulations of harvest

resulting in reduced population size have shown direct negative effects on genetic diversity (Cruse-Sanders et al., 2005). Low levels of allozyme diversity within wild populations have raised concerns regarding the potential for inbreeding depression (Grubbs and Case, 2004; Cruse-Sanders and Hamrick, 2004a).

In a given species, the potential for inbreeding depression depends on the evolutionary history of inbreeding within that species. For plants, the primary determinant of this is the breeding system (Loveless and Hamrick, 1984; Husband and Schemske, 1996). Flowers of *P. quinquefolius* are self-compatible, but previous studies of the reproductive biology of *P. quinquefolius* have found various levels of protandry (Carpenter and Cottam, 1982; Lewis and Zenger, 1983; Schlessman, 1985), which is one mechanism to reduce self-pollination through temporal separation of male and female functions (Bawa and Beach, 1981). Reduced seed set in small experimental populations has been attributed to pollen limitation ostensibly resulting from fewer pollinator visits (Hackney and McGraw, 2001). Estimates of inbreeding coefficients using molecular markers are consistent with high levels of inbreeding by self-pollination or biparental inbreeding within contemporary populations (Grubbs and Case, 2004; Cruse-Sanders and Hamrick, 2004b). But while present levels of inbreeding may be high, the scale of commercial harvest since its inception in the early 1700's suggests that historical population sizes were often much greater than those found today. Therefore, the high level of inbreeding could be a recent phenomenon in evolutionary terms, making inbreeding depression more likely (Lande and Schemske, 1985). Overall, selfing readily

occurs in *P. quinquefolius* as part of its mixed mating system, but the contribution of outcrossing should not be discounted.

Some natural populations of *P. quinquefolius* may be subjected to unusual levels of outcrossing through the introduction of genotypes selected elsewhere. This is possible because in addition to being a highly sought-after wild species, *P. quinquefolius* is widely grown as a cash crop, although cultivated roots are readily discernable from higher-priced wild roots (Carlson, 1986). Cultivation practices for *P. quinquefolius* range in intensity from 100 ha fields where plants are grown under artificial shade (Proctor and Bailey, 1987) to 'wild-simulated' production that can simply be the broadcast of seeds into a forested plot (Beyfuss, 1995). Of intermediate intensity, 'woods grown' can refer to the production of roots in prepared beds under a natural canopy (Beyfuss, 1995). *P. quinquefolius* plants in cultivation produce copious amounts of seeds relative to wild plants, which are frequently sold on the open market (Schluter and Punja, 2000). Cultivated seeds could be introduced to the forests where wild *P. quinquefolius* grows for several reasons: planting could take place by woods grown and wild simulated growers, or managers and harvesters can plant seeds seeking to 'restock' forests where ginseng has seemingly been extirpated (USFWS, 2005). Although *P. quinquefolius* is not considered a domesticated species, there is evidence that important differences have accrued between cultivated and wild populations in the ~120 years of commercial production. Cultivated populations have been shown to be dramatically more diverse (as shown by genetic markers) than wild *P. quinquefolius* populations, which reflects the origin of cultivated stocks from seeds exchanged between growers (Bai et al., 1997; Grubbs and Case, 2004).

Comparisons between wild and cultivated populations have found detectable genetic differences; for example, randomly amplified polymorphic DNA markers have been found unique to plants cultivated in Wisconsin, where most of the *P. quinquefolius* is grown in the United States (Lim, 2004; Schlag, 2004). Although intentional selection efforts are preliminary (Canter et al., 2005), many unintentional selective effects likely result from the interactions with humans in the cultivated and wild environment (Mooney and McGraw, 2007). Unintentional selection has led to profound divergence between domesticated species and their wild relatives in a variety of plant species (Zohary, 2004). Genotypes selected for many generations for success under the high nutrient, low stress conditions of cultivation are likely to be different from those of wild relatives.

If flowering times overlap, hybridization between wild and cultivated plants could occur with several possible outcomes. One potential result could be outbreeding depression as shown by reduced fitness of cultivated-wild hybrids. In the first generation (F1), outbreeding depression may arise from the introduction of cultivation-selected genes that dilute the effects of locally-adapted genes (Montalvo and Ellstrand, 2001; Hufford and Mazer, 2003). Subsequent generations (F2 or later) may show additional evidence of outbreeding depression as recombination breaks down coadapted gene complexes (Lynch, 1991; Fenster and Galloway, 2000; Hufford and Mazer, 2003). Previous studies have found increased performance in F1 hybrids, but reductions in fitness in later generations (Fenster and Galloway, 2000; Hufford and Mazer, 2003). Although these results suggest limitations to examining F1 fitness, the performance of cultivated-wild hybrids in the wild environment would determine if future generations of

hybridization are possible. Alternative to fitness declines in hybrids, genetic swamping may occur if cultivated-wild hybrids outperform local genotypes (Hufford and Mazer, 2003). Given significant fitness advantages, cultivated-wild hybrids could eventually replace local genotypes (Hufford and Mazer, 2003). As a long-lived species, F1 hybrids could presumably persist in populations of *P. quinquefolius* for some time.

Our objective was to assess the first generation consequences of inbreeding and outcrossing for offspring fitness in *P. quinquefolius*. To examine the results of inbreeding, we produced offspring by self-pollinating plants and crossing plants with individuals at two distance classes within wild populations. To assess the consequences of outcrossing with cultivated genotypes, offspring were produced by pollinating flowers of wild maternal plants with pollen from cultivated plants. Progeny resulting from both sets of crosses were planted in natural conditions and their germination, growth and survival was followed for four years. Although additional fitness consequences would likely appear in later generations, the average of 5-10 years it takes for *P. quinquefolius* to reach reproductive maturity made performing subsequent crosses unfeasible. Because *P. quinquefolius* is a long-lived species, we wanted to determine how characteristics observed early in the life-cycle might influence survival over longer timeframes than we were able to observe the offspring of the experimental crosses. For this purpose, we took advantage of monitoring data we collected as part of an ongoing study of eight wild populations in West Virginia, USA.

## Materials and Methods

***Reproductive biology—*** Populations of *P. quinquefolius* are distinctly stage-structured, with individuals being readily classified on the basis of their leaf number. Reproduction in *P. quinquefolius* takes place through seed, with vegetative reproduction being rare in wild populations. Juvenile (2-leaved) plants may or may not be reproductive, whereas 3 and 4-leaved plants are near-universally reproductive. A single immature inflorescence is present on plants upon emergence of aboveground portions in early May. These inflorescences develop into a simple umbel containing several small, hermaphroditic flowers (Schlessman, 1987). Anthesis takes place in mid June and continues as flowers mature centripetally within an inflorescence (Lewis and Zenger, 1983; Schlessman, 1985, 1987). Flowers of *P. quinquefolius* are self-compatible, allowing for pollination to occur both within flowers (autogamy) and within an inflorescence (geitonogamy) (Lewis and Zenger, 1983; Schlessman, 1985). In addition, several generalist pollinators visit inflorescences of *P. quinquefolius*, most importantly syrphid flies and halictid bees (Duke, 1980; Lewis and Zenger, 1983; Schlessman, 1985). Flowers contain a single ovary with 1 to 2 (rarely 3) carpels each (Schlessman, 1985, 1987). A single *P. quinquefolius* flower may produce as many as one seed per carpel, but three-seeded fruits are rarely observed in wild populations (Carpenter and Cottam, 1982; Schlessman, 1985; Schluter and Punja, 2000). Fertilized flowers mature into red fruits by late August to early September, with prevalent population to population variation in ripening phenology (McGraw et al., 2005).

***Study populations***— In early June of 2003, we mapped and marked four populations located in the understory of mixed mesophytic forests outside of Morgantown, West Virginia (Figure 4.1). All populations were initially located by surveys conducted as part of an earlier study of the distribution of *P. quinquefolius* (McGraw et al., 2003). Each population is given an acronym in this publication to protect the details of its specific location (CB, CL, FC1, and FC2). The number of reproductive individuals varied between the four populations (Table 4.1); plants were patchily-distributed across approximately 1 ha areas, as is typical of wild populations of *P. quinquefolius* (Cruse-Sanders and Hamrick, 2004b). Reproductive plants in each population were placed into individual enclosures made of poultry netting to exclude herbivores (e.g. white-tailed deer, *Odocoileus virginianus*). The largest population (FC1) contained 44 reproductive plants, such that we were able to apply both inbreeding and outcrossing pollination treatments.

***Pollination treatments***— We sought to compare offspring of self-pollinations to those created by crosses with plants of differing relatedness to the maternal plant with the inbreeding pollination treatments. To infer relatedness in situ, we used information from previous tests for fine-scale genetic structure in populations of *P. quinquefolius* using allozymes (Cruse-Sanders and Hamrick, 2004b). Spatial distribution is patchy and significant levels of relatedness occur among plants within 2 m of one another for most populations (Cruse-Sanders and Hamrick, 2004b). We measured distances between plants using an electronic distance measuring tool (Sonin Multi-Measure Combo PRO, Sonin, Inc, Charlotte, North Carolina). Few reproductive plants overall were located within 2 m

of each other in our study populations. To accomplish crosses of likely close relatives, plants within a mean of 3.45 m from each other were classified as within the same patch. Plants at greater distances were classified as being in different patches, with the mean distance between plants in different patches 24.24 m. Individuals in populations CB, CL and FC1 were randomly assigned to act as maternal plants and receive one of the three inbreeding pollination treatments: self-pollination, crosses with a plant within their patch and crosses with a plant in a different patch. Unfortunately, population sizes made it unfeasible to perform interpopulation crosses as well. Because of the proximity of flowers within an inflorescence, each inflorescence (one per maternal plant) was assigned a single pollination treatment.

In the outcrossing pollination treatments, we sought to compare the offspring of crosses within the population to those with cultivated plants. Outcrosses within the population were achieved using pollen from other plants in the population, Although distances between plants were not measured. To provide pollen at the two levels of outcrossing with cultivated plants, we obtained reproductively mature plants from cultivated sources in West Virginia (woods grown) and Wisconsin (field grown) as bare roots. Seeds from growers in both Wisconsin and West Virginia are commercially available, so they represent populations that could be used for restoration purposes. Wisconsin was chosen as one source because the majority (>75%) of commercially-cultivated *P. quinquefolius* in the U.S. originates from farms in Wisconsin (Hsu, 2002). The cultivated roots were planted into standard potting soil in 10-inch Deepots (Stuewe and Sons, Corvallis, Oregon). When not being used to pollinate the study populations,

cultivated plants were kept separately in a naturally-shaded location to avoid inadvertent pollen transfer.

***Pollination process***— Prior to the onset of flowering, inflorescences were bagged with small caps made of fine nylon mesh; this was done to exclude natural pollinators, which might visit flowers and deposit pollen. Flowering commenced between June 18$^{th}$ and July 1$^{st}$, and the final flowers within inflorescences opened between July 23$^{rd}$ and 26$^{th}$ in the four populations. As each flower opened, stamens were removed using fine forceps. Pollen was applied by physically dusting the stigmas with an anther obtained from a plant matching the corresponding pollination treatment. For example, stigmas of plants assigned the WV cultivated pollination treatment were dusted with pollen from a stamen of WV cultivated plant. Plants to be self-pollinated were similarly pollinated using this technique; that is, their stamens were removed but were used to dust their own stigma. Populations were visited every 24 to 48 hours during the flowering period to emasculate flowers and apply the pollination treatments. Control plants in each population had their stamens removed without subsequent pollination to test whether pollination occurred before emasculation could take place or if pollen reached the stigma despite bagging.

***Fruit set and planting***— Previous researchers have counted the number of carpels per flower by counting stigmas under magnification, with one stigma per carpel (Schlessman, 1985; Schluter and Punja, 2000). However, differentiation between single stigmas versus stigma lobes proved difficult to determine in situ, so we cannot determine

seed set on the basis of seeds per carpel. The majority of fruits were one-seeded, and no 3-seeded fruits were observed on any plant (Table 4.2). Fruit set was low, with a mean of 18.9% of pollinated flowers producing berries across all pollination treatments. To examine if there were differences in fruit, we compared the proportion of flowers producing berries among pollination treatments using log-likelihood tests (Sokal and Rohlf, 1995).

Fruit maturity can significantly affect germination and ripening phenology varies between populations (McGraw et al., 2005). Individual fruits were collected only upon completion of ripening from August 9$^{th}$ to September 8$^{th}$. We constructed seed cages out of 10 cm sections of 7.5 cm diameter ABS piping with heavy-duty nylon screening attached to the bottom. The cages were filled with soil from the site that was carefully examined to remove any native seeds. Seeds were planted 3 cm deep into their own individual seed cages, which were each labeled with an engraved aluminum nail. The seed cages were then placed within 1 m of the maternal plant to simulate natural dispersal distances. Seed cages were placed within enclosures made out of poultry netting to prevent herbivory of seedlings by white-tailed deer (*Odocoileus virginianus*).

***Germination***— *P. quinquefolius* seeds possess simple, deep morphophysiological dormancy and remain in the soil for at least 20 months before germinating (Anderson et al., 1993; Baskin and Baskin, 1998). Given this dormancy period, the majority of seeds were expected to germinate in 2005, i.e. the second spring following planting. Nevertheless, we monitored the seed cages monthly from May to September of 2004 to

replace the poultry netting enclosures or replant seed cages if disturbed. At the start of May in 2005 and 2006, we checked the seed cages for emergence of seedlings from the soil. In August of 2006, the soil from the seed cages where no seedling appeared in 2005 or 2006 was carefully sifted to recover any remaining seeds. If a seed was present, we tested for viability using a 0.1% tetrazolium solution (Baskin and Baskin, 1998).

***Growth and survival***— We measured stem height (from soil to base of leaf) once emergence of new seedlings was complete in May of 2005. We photographed the leaves of each seedling against a white background. By including a ruler for scale, we were able to convert the pixels of each digital leaf image into an area measurement using NIH Image v.1.63 software (NIH, 2005). The seedlings were monitored bimonthly until September to follow their fates over the 2005 growing season.

In May of 2006, we measured the leaf area and stem height for any new seedlings and re-emerging 1-year old plants using the same techniques as above. In July of 2006 we also estimated chlorophyll content of leaves using a Minolta SPAD 502 Chlorophyll meter (Konica Minolta Group, Hong Kong). We used the leaf area measurements from plants present in both 2005 and 2006 to determine the relative growth rate on a leaf area basis:

$$\mathrm{DRGR}_{\mathrm{LA}} = \frac{\ln A_2 - \ln A_1}{t_2 - t_1}$$

for which $A_2$ is the leaf area in 2006 and $A_1$ is the leaf area in 2005 and the time interval would be one year (McGraw and Garbutt, 1990).

In August 2006, living seedlings were removed from the surrounding soil and separated into root and shoot portions. Sections of lateral root tissue (1 cm long) were tested for mycorrhizal colonization using the Trypan blue staining technique (Phillips and Hayman, 1970). The sections of stained root were examined under a dissection microscope at 60X for the presence of mycorrhizal structures. We also determined root and shoot biomass after drying in an oven at 80°C for 48 hours. Seed cages where seedlings had germinated and apparently 'died' were carefully searched for any remaining root material; if a root had an apical bud present on the rhizome it was considered to be viable.

***Seedlings in wild populations***— To determine the significance of leaf area and stem height measures for longer-term survival, we used demographic data from 8 populations in West Virginia monitored since 2002. Individual plants are measured twice-yearly following the techniques outlined by McGraw and Furedi (2005). New seedlings were identified in these populations by the presence of a persistent seed coat. By following the fate of seedlings in 2002, we determined the relationship of initial seedling stem height and leaf area to survival over four years using logistic regressions (Sokal and Rohlf, 1995). The analyses were repeated for 1-year old plants in 2003 (e.g. new seedlings from previous year) to determine the importance of measures taken at this time for subsequent survival. Using leaf area measures from 2002 and 2003, we were also determined how $DRGR_{LA}$ predicted survival to 2006 using logistic regression (Sokal and Rohlf, 1995).

***Data analysis***— Within the inbreeding and outcrossing studies, data were pooled across populations due to low sample sizes in certain population-pollination treatment combinations. Because of the relatively few seeds (n=33) produced by the inbreeding study, the within and between patch pollination treatments were combined such that subsequent analyses compared seeds produced by self-pollination to those produced by cross-pollination with other plants in the population (regardless of distance). This also allowed the incorporation of seeds produced by the within population crosses for the outcrossing study at the FC1 population. Because no comparable self-pollinated seeds were produced at FC2, seeds produced by the within population crosses for this population were not likewise included.

Germination frequency and year of germination were compared among the seeds produced by the pollination treatments using log-likelihood tests for both the inbreeding and outcrossing studies. For those seedlings that germinated in 2005 or 2006, size measures (leaflet length and width, leaf area and stem height) were compared among pollination treatments using a one-way ANOVA (Sokal and Rohlf, 1995). Survival of seedlings to 1-year old plants in 2006 was compared among pollination treatments using a log-likelihood test. Size measures of 1-year old plants measured in 2006 and their $DRGR_{LA}$ were both compared among pollination treatments using one-way ANOVAs. Finally, we compared root and shoot biomass of seedlings among pollination treatments for both inbreeding and outcrossing studies using one-way ANOVAs (Sokal and Rohlf, 1995). For the outcrossing study, results of one-way ANOVAs showing significant differences were examined further using Tukey-Kramer HSD to detect specific

differences among groups. All statistical tests were completed using SAS JMP version 5.1 (SAS Institute, 2002) and significance was recognized when $p < 0.05$.

## Results

***Fruit set—*** For the control plants, 3.1% and 4.1% of flowers set fruit in the populations used for the inbreeding and outcrossing studies respectively. Self-pollination produced a greater proportion of flowers that formed fruit relative to crosses at either distance within the population ($df = 2$; $\chi^2 = 11.972$, $p = 0.0025$; Fig.4.2 A). There was a trend ($0.05 < p < 0.10$) for the proportion of flowers producing fruit to vary among the outcrossing pollination treatments ($df = 2$; $\chi^2 = 4.734$, $p = 0.0938$; Fig. 4.2 B). Flowers pollinated using WV cultivated pollen tended to have a slightly higher rate of fruit set than those pollinated by either plants within the population or by WI cultivated plants.

***Germination***— Three out of 11 seeds produced by one plant crossed with WV cultivated pollen germinated in May of 2004; however, most germination took place in May of 2005 as expected. In 2005, seeds produced from self-pollination germinated at a rate similar to those produced by crosses within the population ($df = 1$; $\chi^2 = 1.003$, $p = 0.3166$). Likewise, there was no difference in germination rate of seeds produced by the outcrossing treatments ($df = 2$; $\chi^2 = 3.970$, $p = 0.1380$). In May of 2006, two new seedlings appeared, one produced by cross-pollination in the inbreeding pollination treatments and one produced by crosses with WV cultivated plants among the outcrossing pollination treatments. Upon exhuming the seed cages in August 2006, five intact (e.g. endocarp not broken) seeds were recovered from among the 27 cages that showed no

germination in either 2005 or 2006. All intact seeds were the offspring of crosses with WV cultivated plants and were viable.

***Size, growth and survival of experimental seedlings—*** No differences in stem height, leaflet length and width, or leaf area were observed between seedlings produced by different pollination treatments in either the inbreeding or outcrossing studies in the first year following germination. Likewise, there was no difference in survival over the first season of growth (2005) between seedlings produced by self-pollination and those produced by cross-pollination (df = 1; $\chi^2 = 0.292$, p = 0.5891). There were also no differences in survival over the first season among seedlings produced by the outcrossing pollination treatments (df = 2; $\chi^2 = 1.758$, p = 0.4152).

Altogether, most seedlings (83.7%) that survived the first season of growth re-emerged as 1-year old plants in May of 2006; a majority of these plants were 1-leaved, but a few individuals emerged as 2-leaved juveniles. A higher number of offspring produced by crosses with WI cultivated plants emerged as juveniles than offspring produced by either crosses within the population or with WV cultivated plants (df = 2; $\chi^2$ =10.669, p = 0.0048).

Differences in size of plants were apparent in 1-year olds in 2006 that were not observed in the seedlings in 2005; offspring produced by crosses more distant from the wild maternal plant produced larger offspring. In the inbreeding study, 1-year old plants produced by cross-pollination had 44.9% greater leaf area than those produced by self-

pollination (ln transformed; F = 5.4918, p = 0.0308; Fig. 4.3 A). Seedlings produced by crosses with WI cultivated plants had 127.3% greater leaf area than seedlings produced by crosses within the population (square root transformed; F = 4.3810, p=0.0189; Fig. 4.3 B). In the inbreeding study, offspring of cross-pollination also showed 33.0% greater stem height than offspring produced by self-pollination (F = 4.4340, p = 0.0495; Fig. 4.3 C). There were no significant differences in stem height among offspring produced by any of the outcrossing pollination treatments (F = 0.4424, p = 0.6456; Fig. 4.3 D). There was a trend towards higher chlorophyll content in cross-pollinated offspring relative to those produced by self-pollination (F = 3.6795, p = 0.0731; Fig. 4.3 E). No differences in chlorophyll content were observed among offspring produced by the outcrossing treatments (F = 0.8973, p = 0.4174; Fig. 4.3 F).

There were no differences in survival to August 2006 between the inbreeding or among the outcrossing pollination treatments. There was also no difference in relative growth rate on a leaf area basis ($DRGR_{LA}$) between offspring produced by self-pollination or outcrossing (F = 1.4693, p = 0.2411). However, the $DRGR_{LA}$ was significantly higher in the offspring of crosses with WI than either the offspring of crosses within the population or with WV cultivated plants (F = 9.0131, p = 0.0007). Remarkably, one individual produced by crosses with WI cultivated plants flowered and produced a single viable seed.

Most differences observed in $DRGR_{LA}$ were also reflected in the biomass measurements. In the inbreeding study, there were no significant differences in biomass

of roots between offspring produced by self-pollination and cross-pollination (Fig. 4.4 A). Offspring produced by crosses with WI cultivated plants showed 164.8% greater root biomass than outcrossing within the population ($F = 3.7481$, $p = 0.0335$; Fig. 4.4 B). For shoot biomass, no differences were observed between offspring of self-pollination and cross-pollination (Fig. 4.4 C). However, offspring produced by crosses with WV cultivated plants showed a trend towards greater shoot biomass than offspring of crosses within the population or with WI cultivated plants ($F = 2.9369$, $p = 0.0740$; Fig. 4.4 D). Because many roots lacked lateral roots, fewer roots were available for mycorrhizal testing. All of the roots tested ($n = 12$) from offspring produced by either self-pollination or cross-pollination showed evidence of mycorrhizal colonization (data not shown). Despite small sample sizes, seedlings produced by crosses within the population had a significantly higher rate of mycorrhizal colonization (87.5%) than either set of offspring produced by crosses with cultivated plants, WI=16.67% and WV = 42.86% ($df = 2$; $\chi^2 = 7.452$, $p = 0.0241$; Fig. 4.5).

***Size, growth and survival of wild seedlings***— Among the 8 wild populations analyzed, we were able to assess the survival of 78 new seedlings that germinated in 2002. Leaf area of new seedlings was found to be a significant positive predictor of survival to 2006 ($df = 1$; $\chi^2 = 5.0482$, $p = 0.0246$; Fig. 4.6 A). Also, there was a trend suggesting a positive relationship between survival to 2006 and stem height of new seedlings in 2002 ($df = 1$; $\chi^2 = 3.2653$, $p = 0.0708$). Because of 39.7% mortality in the first year following germination, fewer 1-year old plants were available for analysis; however, leaf area was again a significant positive predictor of survival to 2006 ($df = 1$;

$\chi^2 = 4.6462$, $p = 0.0311$; Fig. 4.6 B). No relationship between stem height of 1-year old plants and survival to 2006 was found. $DRGR_{LA}$ measured from 2002 to 2003 was not a significant predictor of survival to 2006 ($df = 1$; $\chi^2 = 0.906714$, $p = 0.3410$).

## Discussion

The pollination treatments in this study simulated fates that *P. quinquefolius* populations could experience: reduction in population size from harvest, deer browse or habitat destruction and the introduction of cultivated plants into wild populations. The inbreeding pollination treatments sought to assess how increased inbreeding in small populations could affect offspring fitness. We wanted to evaluate the potential effects of gene flow from cultivated plants on offspring fitness with the outcrossing pollination treatments.

One of the potential fitness differences between self-pollination and cross-pollination we examined was fruit set. The differences we observed were in contrast to previous studies in *P. quinquefolius*, which have reported equal fruit production between experimentally self-pollinated and cross-pollinated flowers (Lewis and Zenger, 1983; Schlessman, 1985). Our fruit production results need to be carefully interpreted for several reasons. Namely, we were only able to assess fruit set on a per-flower basis, which does not assess the potential of some flowers to produce variable numbers of seeds. Although a majority of fruits we observed were one seeded, these can develop from fruits with a single carpel or through selective post-pollination events in fruits with more than one carpel (Schlessman, 1985). Generally, processes affecting seed set may be

broken down sequentially into variation in pollination (pollen reaching stigmas), fertilization (pollen tube growth through stylar tissue), and seed maturation (development of fertilized ovules) (Lyons et al., 1989). In one previous study of cultivated *P. quinquefolius*, researchers found that variation in the amount of pollen reaching stigmas did influence seed set (Hackney and McGraw, 2001). All flowers in this study were consistently hand-pollinated upon anthesis, thus pollination is assumed to be equal among treatments. Differences in pollen tube formation and growth would reflect maternal and paternal interaction, whereas fruit set could be attributed to maternal resource partitioning or it may be considered an early-life history fitness measure for offspring (Lyons et al., 1989; Waser and Price, 1993). Because pollen tube growth and selective embryo abortion were not determined in situ, we are limited in how we can interpret differences in fruit set with respect to the influence of inbreeding depression. Nevertheless, our results from self-pollinated flowers support previous findings demonstrating the success of this mechanism for reproductive assurance in this species.

Whereas fruit and seed production is an interacting mixture of maternal, paternal and offspring fitness, the germination of seeds allows us to evaluate offspring fitness more directly. Regardless of the pollination treatment that produced them, most seeds germinated after remaining dormant through two winters, which was the expected pattern (Baskin and Baskin, 1998; McGraw et al., 2005). The lack of inbreeding depression in germination could be a result of previous generations of close inbreeding that effectively purged any deleterious alleles that would act at this stage in the life cycle (Husband and Schemske, 1996; Schierup and Christiensen, 1996). Alternatively, the lack of differences

between selfed versus coss-pollinated seeds in terms of germination may be explained by the relatively benign planting environment. Specifically, although seeds were planted in natural soil, they were planted at an optimal depth for germination of *P. quinquefolius* (McGraw et al., 2005). Previous studies have suggested that favorable environments may mitigate the effects of inbreeding (Dudash, 1990; Heschel and Page, 1996)

In the year of germination, offspring were similar in regard to survival and size, but as of the second year of growth differences in these traits emerged. In some previous studies, statistically significant differences among offspring produced by controlled crosses appeared only later in the life cycle of perennial plants (Wolfe, 1993; Waser and Price, 1994; Kephart et al., 1999; Luitjen et al., 2002). Generally, differences are likely to increase with time due to the expression of the offspring genome overriding maternal genetic and environmental variation (Wolfe, 1993; Husband and Schemske, 1996). In our test for inbreeding depression in *P. quinquefolius*, we found differences in leaf area and stem height in year two of growth, but the lack of differences in biomass suggests that size differences did not lead to early differences in carbon acquisition. However, the data collected from the eight wild populations indicates that initial leaf area differences could ultimately lead to survival differences over longer time frames. To some extent, these results follow from theory that inbreeding depression in self-fertile species would be attributed to many loci of small effect, rather than easily purged, highly deleterious recessives (Charlesworth and Charlesworth, 1987; Scheirup and Christiansen, 1996). In a majority of studies of self-fertile species reviewed by Husband and Schemske (1996), inbreeding depression was exhibited in growth and reproduction. Alternatively, if

populations that become fixed for deleterious alleles of small effect through isolation and drift, inbreeding depression may only become apparent if among population crosses are performed; that is, offspring of crosses within populations may not show any inbreeding relative to one another as they would be similarly inbred (Keller and Waller, 2002). Nevertheless, we see some evidence of inbreeding depression, suggesting that complete fixation of deleterious alleles has not occurred in these wild populations. One reason for inbreeding depression is that historical population sizes were likely much larger, such that outcrossing frequency was much greater in the evolutionary past of *P. quinquefolius*.

Differences that result from comparing offspring produced by self-pollination and outcrossing within the population are best interpreted with regards to the dynamics of mating system evolution. Plants like *P. quinquefolius* with a mixed mating system seemingly defy genetic theory which predicts that species should be either entirely self-fertilizing or outcrossing (Lande and Schemske, 1985). The prevalence of mixed mating systems reflects the many environmental selective pressures governing reproduction, rather than inbreeding depression alone (Goodwillie et al., 2005). One of the main factors recognized to promote mixed mating systems is the reproductive assurance guaranteed by self-compatibility when pollinators or non-self pollen are scarce (Jain, 1976). Indeed, there is some evidence that *P. quinquefolius*, although entirely self-compatible, may suffer from pollen limitation (Hackney and McGraw, 2001). If outcrossing opportunities are limited, self-pollination can be selectively favored, even if inbreeding depression in the offspring is considerable (Herlihy and Eckert, 2002). The evidence we have for inbreeding depression in *P. quinquefolius* despite high selfing rates

is similar to that observed for *Aquilegia canadensis*, another forest perennial with small contemporary population sizes (Herlihy and Eckert, 2002, 2004). Although self-fertilization can achieve reproductive assurance through seed production when pollinators are scarce for *A. canadensis*, inbreeding depression limits the value of seeds and offspring produced this way (Herlihy and Eckert, 2002). For a long-lived species like *P. quinquefolius*, production of selfed seeds may be a way to 'wait out' adverse years when non-self pollen is limited. Such temporal variation has been a significant factor favoring the stabilization of mixed mating systems in evolutionary models (Goodwillie et al., 2005). However, we also see differences in offspring quality between self-pollination and outcrossing indicative of some cost to this reproductive assurance.

Differences in offspring quality between cross-pollination within the population and with cultivated plants must be interpreted with care. In a different context, the increased leaf area and root biomass of offspring of Wisconsin cultivated plants would appear as positive fitness indicators, suggesting the beneficial effects of outcrossing at this scale. Previous investigations of outbreeding depression have found increased performance of first generation intraspecific hybrids relative to parents; but in later generations, the fitness of hybrids can decrease, putatively owing to the breakdown of co-adapted gene complexes (Hufford and Mazer, 2003). In *Chamaecrista fasiculata*, F1 hybrids showed enhanced performance, but some F2 and F3 hybrids showed evidence of decreased fitness relative to the parental population (Fenster and Galloway, 2000). Given the typical pre-reproductive period for *P. quinquefolius* of 5-10 years, we cannot assess what fitness differences may occur in subsequent generations. With our results for *P.*

*quinquefolius*, the achievement of large leaf area of cultivated-wild hybrids may reflect important genetic differences between cultivated and wild plants. The increased leaf area is largely due to the higher frequency of 2-leaved individuals among offspring of Wisconsin cultivated plants. Notably, previous demographic studies of *P. quinquefolius* in the wild have shown that plants reach the 2-leaved stage after three years or more (Lewis and Zenger, 1982; Charron and Gagnon, 1991; Anderson et al., 1993; McGraw and Furedi, 2005). However, *P. quinquefolius* plants in cultivation after three years typically have three or more leaves and produce copious amount of seeds (Hughes and Proctor, 1981; Proctor and Bailey, 1987; Schluter and Punja, 2000; Hsu, 2002). Cultivated populations are propagated from seeds collected from plants prior to harvest, which can occur as early as after just two years of growth (Proctor and Bailey, 1987; Whitbread et al., 1996; Schluter and Punja, 2000; Hsu, 2002); this practice could putatively select for rapid achievement of the size necessary for reproductive maturity. The techniques and environment of cultivation have been long recognized to cause changes in the life-histories of formerly wild species (Darwin, 1882).

Rapid growth of cultivated genotypes may be achieved at a cost to fitness under stressful natural conditions. Specifically, fewer plants produced by crosses with cultivated plants showed mycorrhizal colonization in comparison to the offspring produced by crosses within the populations. Plants may be investing resources into leaf area and root biomass at the expense of allocation to recruit and sustain mycorrhizal fungi (Marschner, 2003). Early recruitment of mycorrhizal fungi may confer benefits later in life as resource demands increase or as soil nutrient pools are depleted (Treseder, 2004).

One of the prominent features of cultivation of *P. quinquefolius* is the application of fungicides to prevent diseases, insecticides to prevent herbivory and the administration of fertilizers (Proctor and Bailey, 1987). Although mycorrhizal associations occur in cultivation (Whitbread et al., 1996), high nutrient availability (specifically phosphorous) has been shown in numerous species to decrease plant investment in mycorrhizal fungi (reviewed in Treseder, 2004). Mycorrhizae may be less important in the cultivated environment than in low nutrient forest soils. Rapid growth may similarly occur at the expense of resistance to insect herbivory and fungal diseases. For example, ginsenosides—a class of saponins found in *P. quinquefolius*— have been shown to be important antifungal agents against ginseng-specific diseases (Nicol et al., 2002); but like other carbon-rich defense compounds, ginsenosides would be produced at the expense of allocation to growth. The accelerated life cycle of cultivated genotypes may have negative fitness effects over longer time periods than observed in this study through the cumulative effects of disease and environmental stress.

From our results we see ecologically important differences conferred by cultivated genes that appear to increase fitness, albeit in the short term for the first generation. The success of our crosses demonstrates that there is no barrier at this stage to gene flow if cultivated individuals were introduced into wild populations (given flowering times coincide). Although it may appear that we are detecting the relief of inbreeding depression—which would be beneficial to population survival— we should emphasize that such rapid growth has not been observed in our monitoring of thousands of plants over several years in the wild (Mooney and McGraw, unpublished data). Our

results illustrate an important caveat to reintroduction and genetic rescue attempts using cultivated source material (Storfer, 1999; Hufford and Mazer, 2003; Tallmon et al., 2004). Similar issues have been addressed in the context of hybridization between wild and weedy species (Levin et al., 1996) and gene flow between wild and cultivated species (Ellstrand et al., 1999; Ellstrand, 2003). Gene flow can result in extinction through demographic swamping if hybrids out perform parental populations (Levin et al., 1996; Wolf et al., 2001; Haygood et al., 2003). Likewise, *P. quinquefolius* populations would be at risk for extinction via genetic assimilation, in which cultivated genes replace wild genes if they confer elevated fitness (Levin et al., 1996; Haygood et al., 2003). This issue has become increasingly important due to concerns about the escape of transgenes from genetically modified crops into wild or weedy relatives (Lu and Snow, 2005; Chapman and Burke, 2006). Recent application of genetic engineering to *P. quinquefolius* has produced plants that express antifungal proteins from *Oryza* species (Chen and Punja, 2004), adding to the potential consequences of outcrossing if transgenic plants become widely adopted.

Altogether, we have examined both the consequences of inbreeding and outcrossing with cultivated genotypes for *P. quinquefolius*; however, anthropogenic change worldwide is likely increasing the instances where plant species simultaneously experience small population size and gene flow from relatives in cultivation. For plants like *P. quinquefolius* with a mixed mating system, self-pollination can offer benefits regarding reproductive assurance when outcrossed pollen or pollinators are limited. However, evaluations of reproductive success in such species need to account for quality

of offspring as well, since inbreeding depression can occur even in species that are readily self-fertile. Our results from outcrosses with cultivated genotypes confirm that the precautionary principle should be applied when wild populations of rare species interact with their cultivated counterparts. If the accelerated growth of cultivated-wild hybrids persists to later generations, the integrity of *P. quinquefolius* populations in the wild would be threatened by restoration efforts involving cultivated source material. Overall, we see that anthropogenic influences on population size in either direction can lead to potentially far reaching consequences for plant species.

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Table 4.1: Number of *Panax quinquefolius* plants in each of the six pollination treatments among four study populations outside of Morgantown, West Virginia USA. Individual plants possessed a single inflorescence which was assigned a single pollination treatment. The numbers of individual flowers are indicated in parentheses.

| *Population* | *Pollination Treatments* | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Inbreeding* | | | *Outcrossing* | | |
| | Control | Self-pollinated | Within cluster | Between cluster | Within population | WV cultivated | WI cultivated |
| CB | 1(4) | 6(26) | 7(35) | 9(43) | -- | -- | -- |
| CL | 2(9) | 4(37) | 6(28) | 9(50) | -- | -- | -- |
| FC1 | 5(27) | 2(12) | 5(20) | 7 (38) | 12(55) | 11(57) | 7(49) |
| FC2 | 4(26) | -- | -- | -- | 3(30) | 8(64) | 5(46) |

Table 4.2: Numbers of one-seeded, two-seeded and total seeds produced among four populations of *Panax quinquefolius* located outside of Morgantown, WV.

| *Population* | *One-seeded fruits* | *Two-seeded fruits* | *Total seeds* |
|---|---|---|---|
| CB | 11 | 0 | 11 |
| CL | 8 | 3 | 11 |
| FC1 | 28 | 12 | 40 |
| FC2 | 26 | 11 | 37 |

Figure 4.1: Map of eastern United States showing location of cultivated plants used as pollen donors from farms in Marathon County, Wisconsin and Preston County, West Virginia. The inset maps show locations of eight wild populations where seedlings were monitored from 2002 to 2006 within West Virginia and locations of experimental populations where controlled pollinations were performed near Morgantown, WV. The maps are modified from those available at http://nationalatlas.gov.

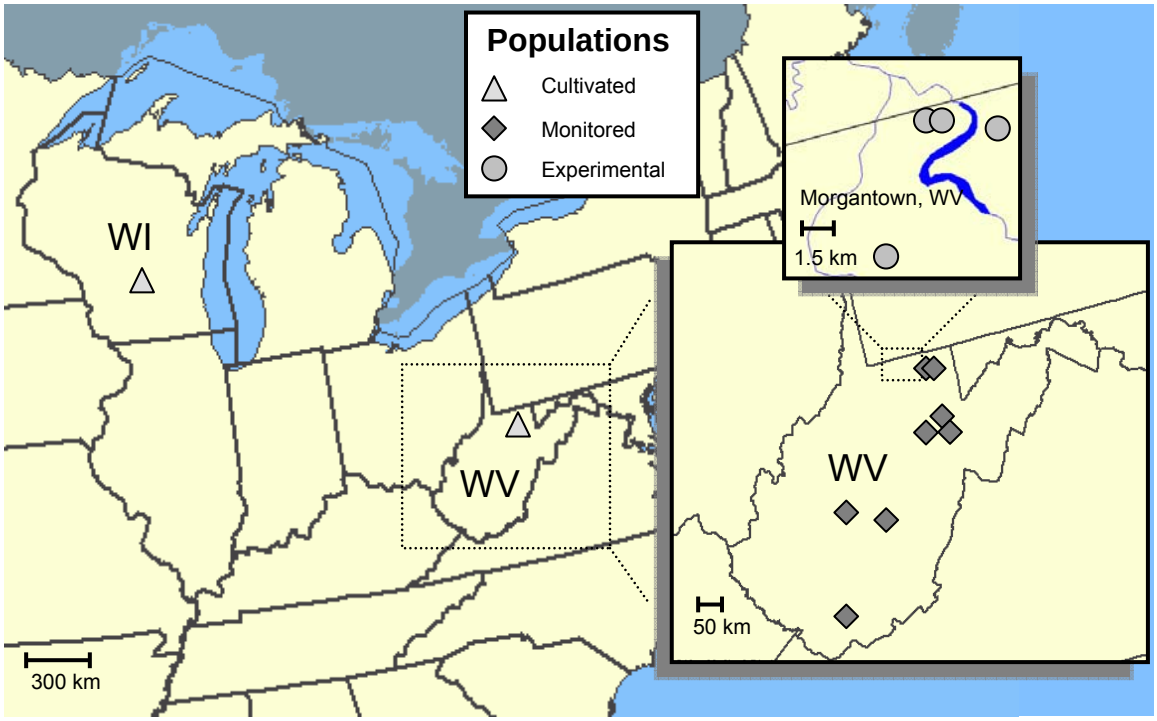


Figure 4.2: Fruit set measured as the percent of *Panax quinquefolius* flowers producing seed in August 2005 for: (A) Inbreeding pollination treatments and (B) Outcrossing pollination treatments.

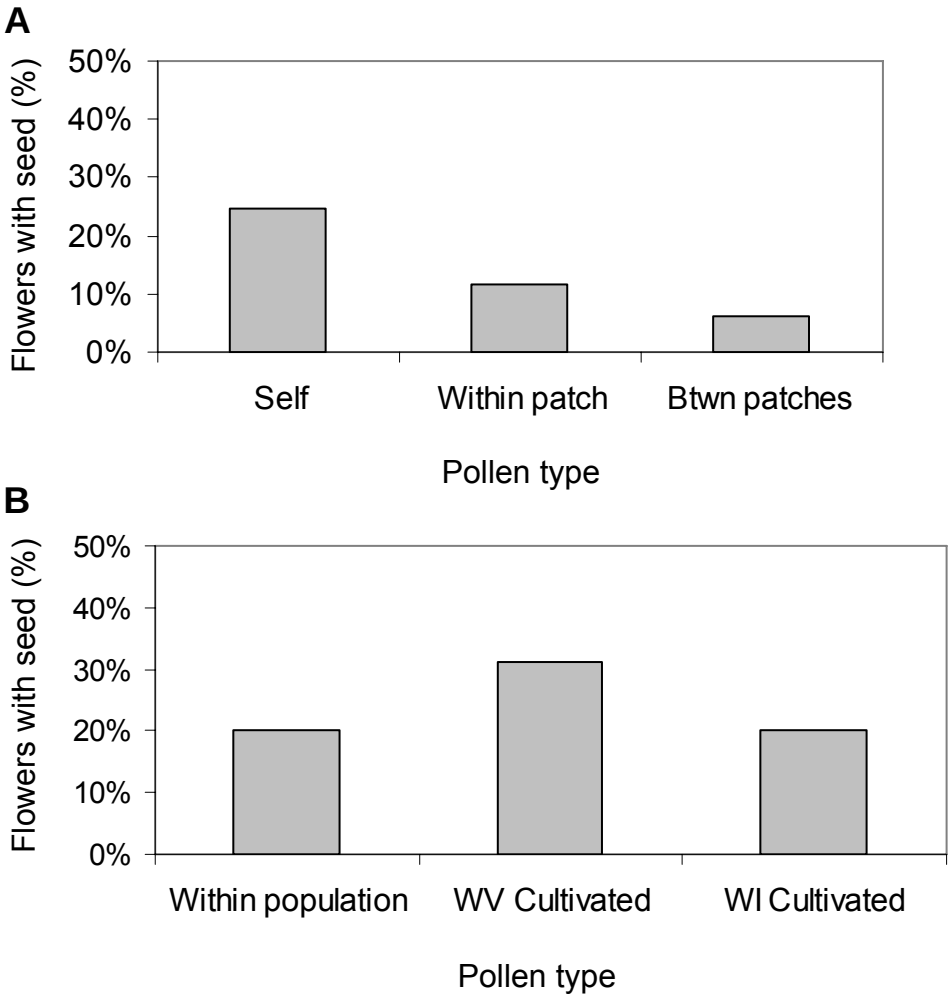


Figure 4.3: Leaf area of 1-yr old *Panax quinquefolius* plants produced by: (A) Inbreeding pollination treatments and (B) Outcrossing pollination treatments. Stem height of plants produced by: (C) Inbreeding pollinations treatments and (D) Outcrossing pollination treatments. Chlorophyll content of plants produced by (E) Inbreeding pollination treatments and (F) Outcrossing pollination treatments. Data are shown as back-transformed means where appropriate. Error bars indicate back-transformed standard errors where appropriate. Letters above bars show differences determined by Tukey-Kramer HSD procedure, superscript "t" indicates trend.

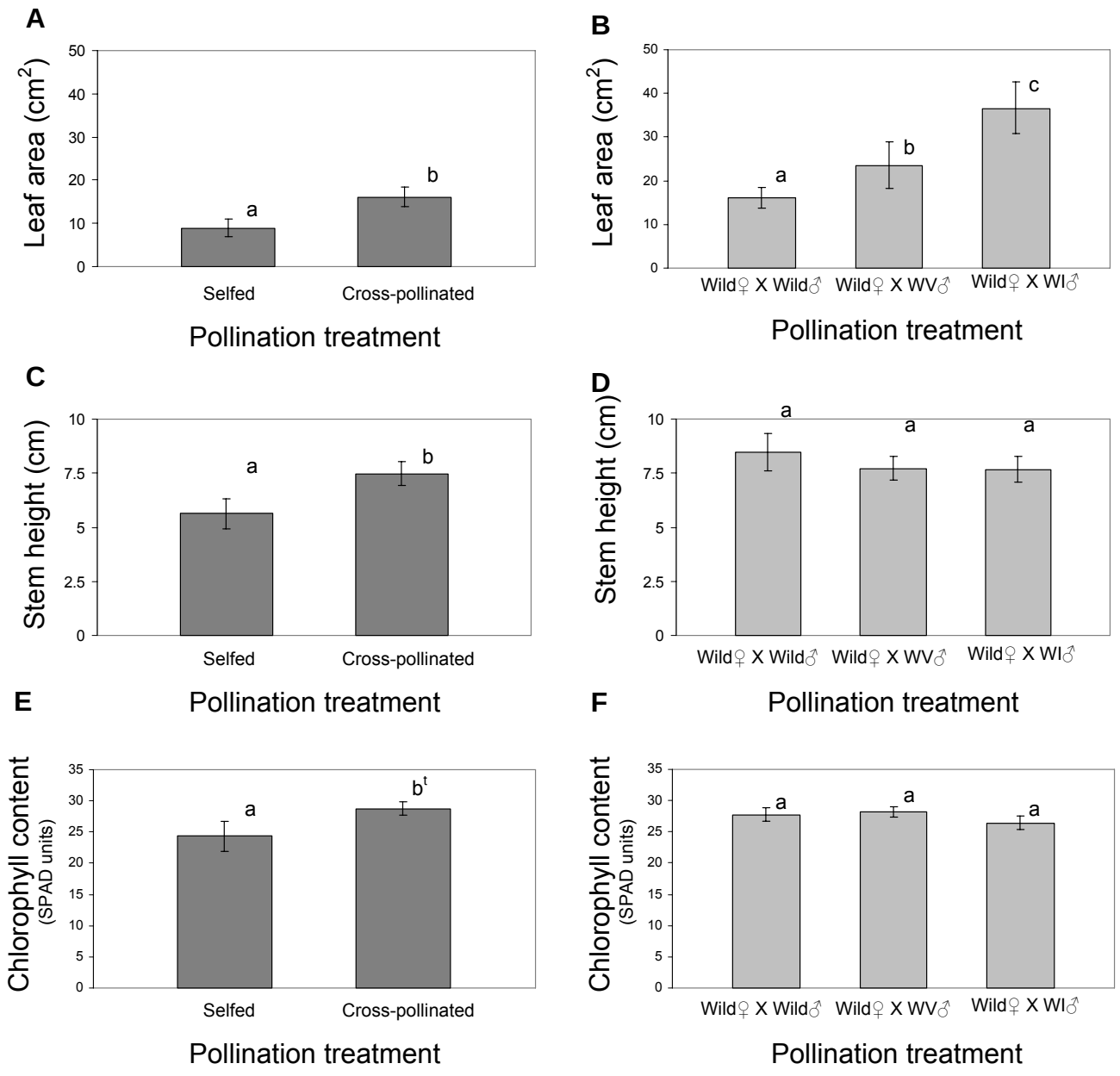


Figure 4.4: Root biomass of 1-yr old *Panax quinquefolius* plants produced by: (A) Inbreeding pollination treatments and (B) Outcrossing pollination treatments. Data are shown as back-transformed means where appropriate. Error bars indicate back-transformed standard errors where appropriate. Letters above bars show differences determined by Tukey-Kramer HSD procedure, superscript "t" indicates trend.

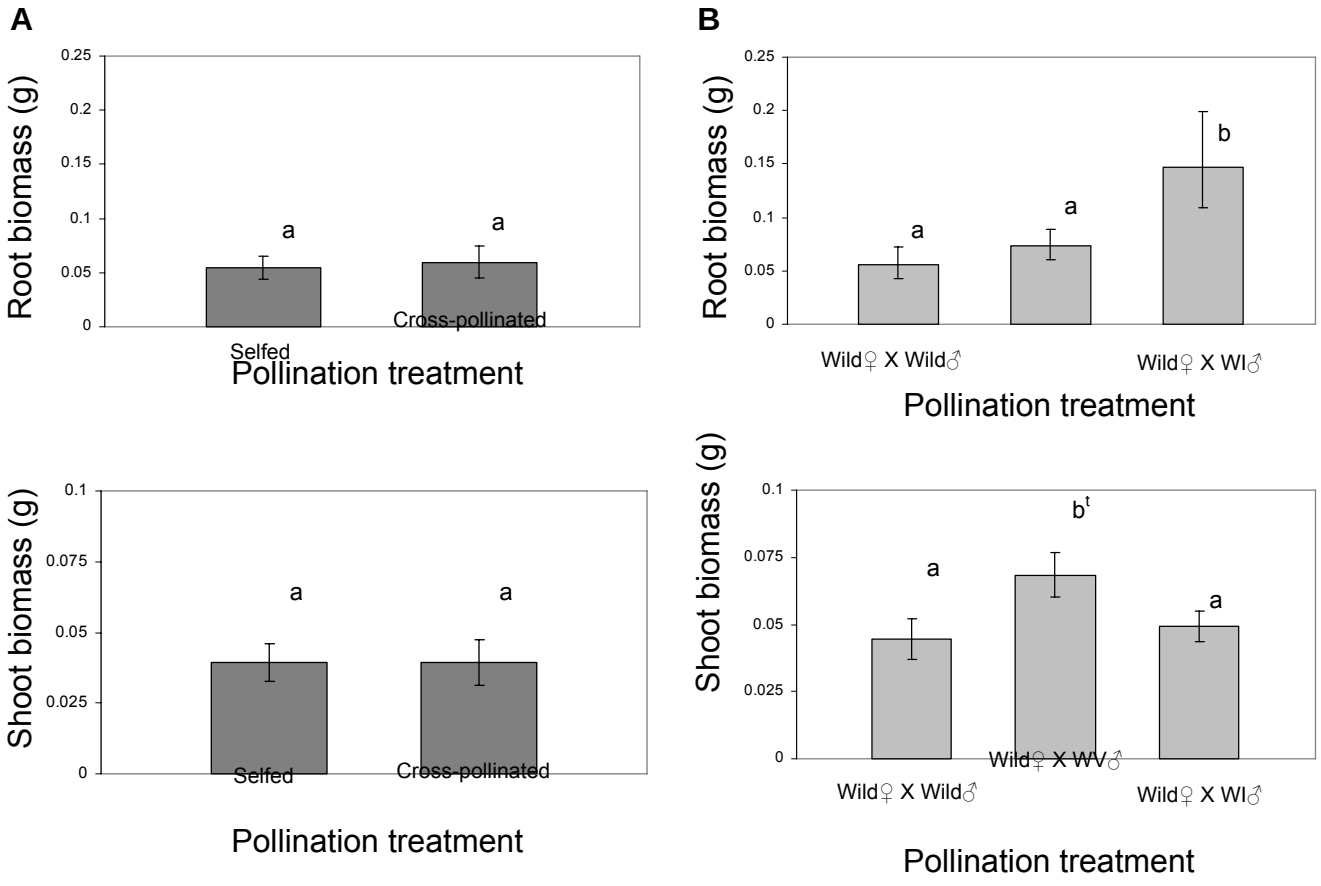


Figure 4.5: The frequency of mycorrhizal colonization in roots of 1-year old *Panax quinquefolius* plants produced by outcrossing pollination treatments.

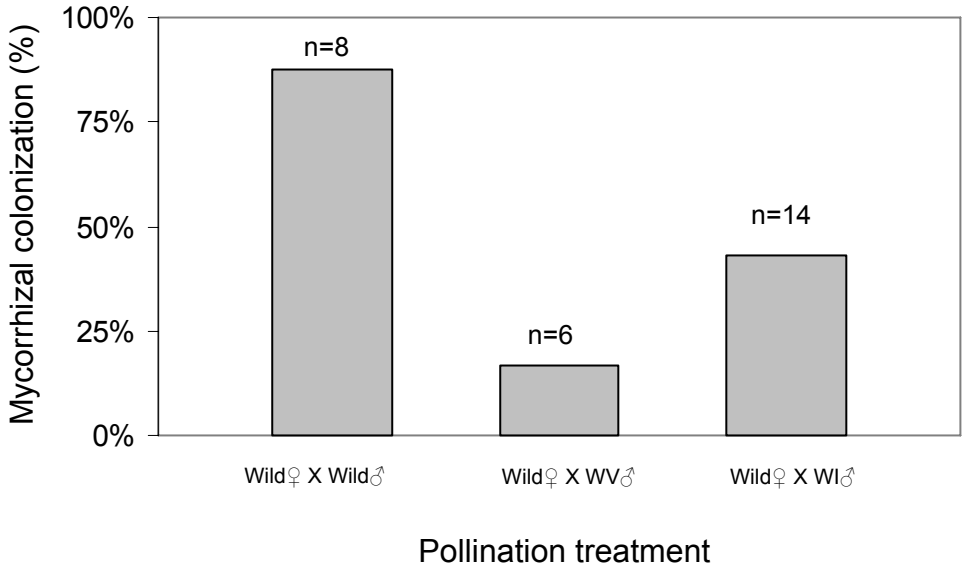
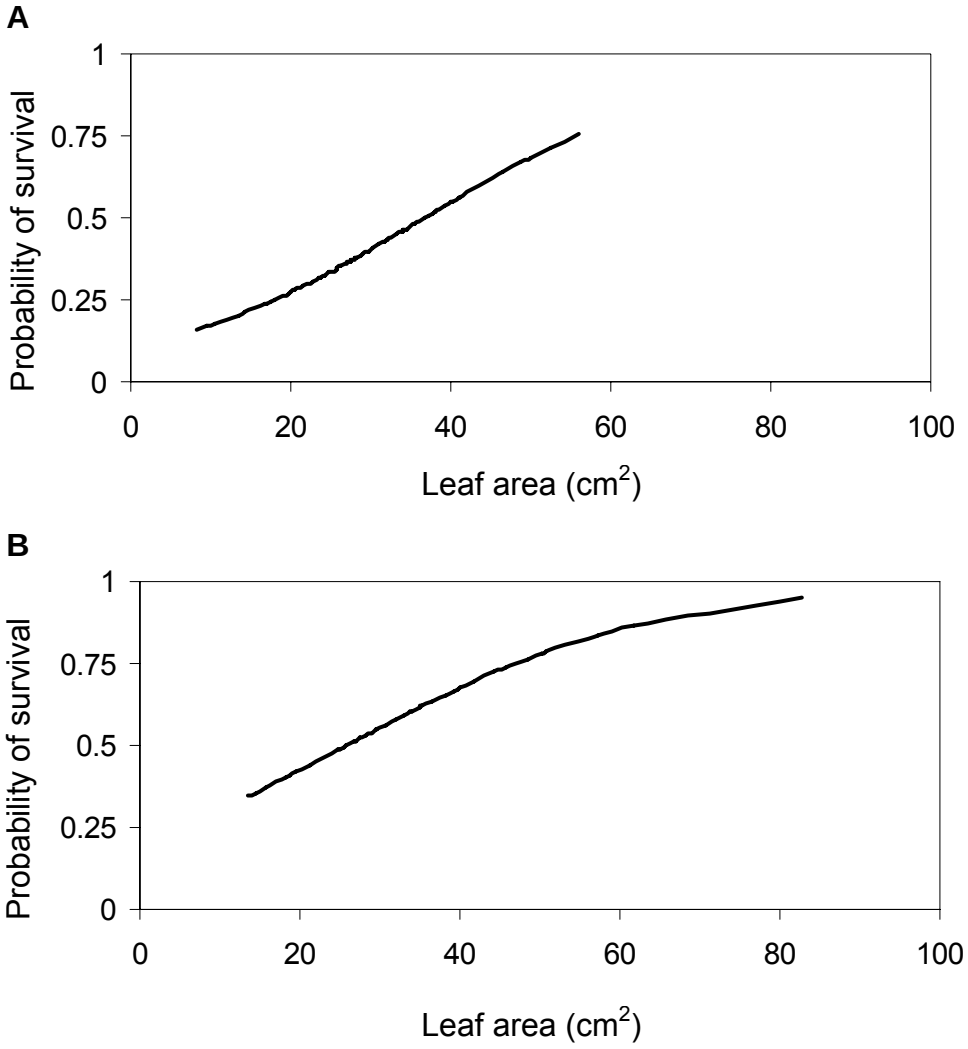


Figure 4.6: Relationship between survival to 2006 and leaf area of (A) New seedlings in 2002 and (B) 1-year old plants in 2003 observed in 8 natural populations of *Panax quinquefolius* in West Virginia. Curves generated from logistic regressions.



# CHAPTER 5

## The influences of genetic diversity, harvest pressure and population size on population growth rate of the rare plant American ginseng, *Panax quinquefolius*

**Abstract**

In terms of their importance for population persistence, the effects of inbreeding and genetic drift versus environmental and demographic stochasticity vary among species. We sought to evaluate the relationship of genetic diversity to population growth rate for *Panax quinquefolius* (American ginseng), a wild-harvested perennial plant. To this end, we conducted a multi-year demographic study of 18 populations of *P. quinquefolius* in 4 states. A seed cage study allowed us to incorporate age-structured seed bank dynamics with stage-structured demography into transition matrix models. From the product of the observed matrices, we determined a multiyear growth rate ($\lambda$) for each population. Along with growth rate, we also characterized the average panmictic heterozygosity (*hs*) of each population using RAPD variation. Because of harvest's influence on population growth rate, size and genetic diversity, we also incorporated an estimate of harvest pressure. We used path analysis to model the hypothesized relationships among harvest index (HI), population size ($N_e$), genetic diversity (*hs*), and their direct and indirect effects on population growth rate ($\lambda$). Harvest index had a negative influence on population growth rate ($P_{HI,\lambda} = -0.488$, $p = 0.028$). Harvest also had indirect effects on population growth rate through its effects on population size ($P_{HI,Ne} = -0.328$, $p = 0.055$), and in turn, genetic diversity. Genetic diversity had a positive influence on population growth rate ($P_{hs,\lambda} = 0.0252$, $p = 0.060$). Overall, the results from the path analysis support the importance of both stochastic environmental events (harvest) and genetic diversity for population growth in *P. quinquefolius*.

## Introduction

A central concept of the field of conservation genetics is that genetic diversity can be an important determinant of population survival (Frankham et al. 2002, Ouborg et al. 2006). Plant species of conservation concern are often characterized by small population sizes, theoretically increasing the likelihood of inbreeding and genetic drift, collectively known as genetic stochasticity (Barrett and Kohn 1991, Ellstrand and Elam 1993). Both inbreeding and drift contribute to the loss of genetic diversity. Over the long term, low diversity can theoretically decrease population survival if it constrains adaptation to environmental change (Huenekke 1991, Ellstrand and Elam 1993). In the near term, both drift and inbreeding can negatively influence population vital rates. Genetic drift can lead to the fixation of deleterious alleles or loss of beneficial alleles (Barrett and Kohn 1991). Inbreeding can have negative consequences if inbreeding depression occurs, i.e., inbred individuals show reduced fitness (Keller and Waller 2002). Of significance to conservation, inbreeding depression is most likely in species where small population sizes are a recent phenomenon, such that deleterious alleles have not been purged by natural selection (Lande and Schemske 1985, Charlesworth and Charlesworth 1987). Reduced genetic diversity has increased extinction probability for experimental (Newman and Pilson 1997) and simulated (Brook et al. 2002) plant populations by negatively affecting population vital rates.

In light of the potential consequences, efforts to characterize the genetic diversity of rare species have proceeded alongside other measures of population viability (Booy et al. 2000, Oostermeijer et al. 2003, Spielman et al. 2004). Most studies have relied on

neutral genetic markers to assess a variety of diversity statistics (Hansson and Westerberg 2002). Generally, interpretations of these results have invoked the idea that heterozygosity and equivalent diversity estimates are indicators of genome wide variability (Milligan et al. 1994, Hansson and Westerberg 2002). Consequently, low diversity measures mean that the studied populations could be at risk for inbreeding depression or the negative effects of drift (Milligan et al. 1994). The importance of genetic stochasticity when populations are faced with threats like habitat destruction, overexploitation and invasive species remains a subject of debate (Spielman et al. 2004, Ouborg et al. 2006). Some have maintained that populations of threatened species are more likely to go extinct through environmental stochasticity before genetic problems take effect (Lande 1988, Schemske et al. 1994, Caro and Laurenson 1994). However, these extinction processes are not mutually exclusive, and the interplay between environmental, demographic and genetic stochasticity may create what Gilpin and Soulé (1986) termed an 'extinction vortex.'

Several studies of rare plants have sought to separate the contribution of genetic diversity to population fitness components (Oostermeijer et al. 1994, Ouborg and van Treuren 1995, Fischer and Matthies 1998, Lammi et al. 1999, Buza et al. 2000, Luitjen et al. 2000, Vergeer et al. 2003). The methods these studies used were similar: (1) seeds were collected from populations and then grown in a greenhouse or field plot, (2) genetic variation of the population was determined, and (3) several measures of fitness were taken, e.g. germination rate, to assess population performance. By growing seeds in a common environment, the authors account for the influence of environmental variation;

fitness differences among populations explainable by genetic diversity gauges the effects of inbreeding and genetic drift. In the earliest of these studies, Oostermeijer and colleagues (1993) examined the rare perennial plant *Gentiana pneumonanthe* and found that diversity of isozyme loci was positively correlated with performance of adults. Similar results were found in the short-lived *Gentianella germanica* (Fischer and Matthies 1998), but other studies found that offspring performance was independent of population diversity (Ouborg and Van Treuren 1995, Lammi et al. 1999, Luitjen et al. 2000, Buza et al. 2000). The lack of correlation between allozyme heterozygosity and fitness components led the authors to conclude that small populations were not affected by inbreeding or drift. However, the relatively benign greenhouse or field plot could mask inbreeding depression that is apparent in the native environment (Dudash 1990, Heschel and Paige 1995, Husband and Schemske 1996). Most of the studies showed that smaller populations had reduced genetic diversity, but the consequences for population performance in the wild remain unclear.

Some previous studies of rare or threatened plants have measured population performance in the wild and found significant relationships with diversity (Menges and Dolan 1998, Schmidt and Jensen 2000, Vergeer et al. 2003, Dittbrenner et al. 2005). These studies have found positive relationships between genetic variation and population growth rate or its constituents, even when measured within the background of environmental variation. Ideally, experimental studies would be performed where genetic variation, population size or density would be independently manipulated as exemplified by the work of Newman and Pilson (1997). Many rare plants are simply too

scarce or produce too few propagules to make experimental approaches to relating genetic diversity and demography practical (Oostermeijer et al. 2003).

Like other plant species of conservation concern, populations of *Panax quinquefolius* (American ginseng) in the wild face several threats: most notably, browse by overabundant white-tailed deer (McGraw and Furedi 2005) and commercial harvest (Nantel et al. 1996, Van der Voort and McGraw 2006). Harvest can have long-term effects on population structure for many years post-harvest (Van der Voort et al. 2003). The effects of harvest on population growth rate will depend on its intensity (Nantel et al. 1996, Van der Voort and McGraw 2006). Harvest also reduces genetic diversity: simulated removal of 10 to 50% of the legally-harvestable plants in a population can result in genetic diversity declines and loss of allelic richness (Cruse-Sanders et al. 2005). Surveys of diversity with allozymes have shown low levels of diversity within and substantial divergence between contemporary populations (Grubbs and Case 2004, Cruse-Sanders and Hamrick 2004). The low levels of diversity create concerns about how genetic effects could additionally limit population recovery from harvest.

The susceptibility of populations to the negative effects of drift and inbreeding depression is generally a function of their evolutionary history. Recurring bottlenecks in populations can theoretically eliminate deleterious recessive alleles (Byers and Waller 1999, Keller and Waller 2002). The effectiveness of such purging is reduced in small populations ($N_e$<100), which are sizes typical of rare or threatened plants (Byers and Waller 1999). Although wild-harvest has been occurring since the 18$^{th}$ century (Carlson

1986), small population sizes are likely a recent phenomena in *P. quinquefolius*. Deer herds have increased over the last quarter-century, making their effects in terms of reducing population sizes relatively recent (McGraw and Furedi 2006). Breeding system also theoretically predicts the susceptibility of a species to inbreeding depression (Lande and Schemske 1985). *P. quinquefolius* exhibits a mixed mating system; several generalist pollinators visit the self-compatible, hermaphroditic flowers (Duke 1980, Schlessman 1985). Mixed mating systems allow selfing to provide reproductive assurance when outcross pollen is limited, even if inbreeding depression occurs (Goodwillie et al. 2005). Altogether, neither the evolutionary history nor the breeding system of *P. quinquefolius* discount the possibility for negative impacts from genetic stochasticity.

Our objective was to assess how population size, harvest pressure and genetic diversity may influence population growth rate of *P. quinquefolius*. To this end, we collected demographic data from 18 populations of *P. quinquefolius* that yielded multiyear growth rates and population sizes. For a neutral measure of genetic diversity, we characterized the populations using random-amplified polymorphic DNA (RAPD) variation. To account for the particular impacts of harvest, we calculated an index of harvest pressure for each population. Given the interrelated nature of population size, harvest pressure and genetic diversity, we chose a path analytical approach to evaluating the strength of their direct and indirect influences on population growth rate.

## Materials and Methods

### Study Species

American ginseng, *Panax quinquefolius* L. (Araliaceae), is an uncommon to rare herbaceous perennial of the eastern deciduous forest understory (Gleason and Cronquist 1991, Anderson et al. 1993, McGraw et al. 2003). Population sizes encountered in the wild are rarely greater than 100 plants, as harvest, habitat degradation and increased deer browse have all contributed to population decline (Hu et al. 1980; Carpenter and Cottam 1982; Lewis and Zenger 1982; Lewis 1984; Lewis 1988; Charron and Gagnon 1991; Anderson et al. 1993; McGraw et al. 2003; McGraw and Furedi 2005). The life cycle of *P. quinquefolius* is made up of distinctive stages: seedlings consist of a single trifoliate leaf, and the size and number of leaves typically increase with the age (Carpenter and Cottam 1982; Lewis and Zenger 1982; Charron and Gagnon 1991; Anderson et al. 1993). Plants produce an inflorescence after a pre-reproductive period of three to seven years (Charron and Gagnon 1991; Anderson et al. 1993). Inflorescences of *P. quinquefolius* are composed of self-compatible flowers, and outcrossing may be facilitated by several generalist pollinators (Duke 1980, Lewis and Zenger 1983, Schlessman 1985). *P. quinquefolius* seeds exhibit morphophysiological dormancy and remain in the soil for at least 18-22 months before germinating in spring (Baskin and Baskin 1998).

### Demographic Data

***Population censuses—*** We censused eighteen naturally occurring populations in four states (IN, NY, VA & WV). The populations were located through field surveys, consultations with managers or local botanists. The onset of censusing varies among

populations based on when they were initially located (Table 5.1). Populations were defined as contiguous clusters of plants within 50m of one another; this definition is based on experimental data showing significant declines in pollen movement past this point (Hackney 1999). Once located, we searched the area for all plants and marked them following the techniques of McGraw and Furedi (2005); that is, plants were cryptically marked and located with the aid of a 'Phototrail.'

Censuses took place upon emergence in May or June and again once seed production was complete in August or September. During the May-June census, we counted the number of leaves on each plant, determined if the plant was reproductive, and measured the length and width of longest leaflet of each leaf. Also at this time, we recorded any new seedlings, which could be readily identified by an attached seed coat. We recorded the production of seeds during August-September census. The fate of missing plants was carefully assessed during both censuses. Often leaves would be missing, but a partial stem and root would remain, indicating browse by white-tailed deer (McGraw and Furedi 2006). Missing plants would be sometimes accompanied by evidence of disturbance (e.g. tree fall or rodent tunneling) or harvest, such that the affected plant would be considered dead. Harvest was discernable from these other types of disturbance by shallow holes at the plant's location.

***Seed bank dynamics—*** Because seeds of *P. quinquefolius* have shown long term dormancy in previous studies (Van der Voort and McGraw 2006), we experimentally measured seed bank dynamics using woods-grown seeds obtained from a single ginseng

farm in West Virginia. In 2002, we dispersed seeds during the August-September census at each of the 14 populations censused at that time. The seeds were contained within twelve cages constructed of a 15cm section of 5 cm diameter ABS pipe with fine nylon screening attached to the bottom. Each of these seed cages contained 50 *P. quinquefolius* seeds planted in soil from the site, and the entire cage was buried level with the soil surface.

In May of 2003, 36 total cages were exhumed from 12 populations (3 per population) to estimate survival of dormant seeds nine months after dispersal. Viability was tested by laterally slicing seeds to expose the embryo, and then incubating seeds in a 0.1% tetrazolium solution for 24 hours without light. We considered the seeds viable if the embryos stained pink (Baskin and Baskin 1998). The surviving proportion in each seed cage was determined by dividing the number of viable seeds by 50 (the number initially dispersed). In May of 2004, 38 cages were removed from 13 populations to measure germination rates and dormancy at 21 months. The germination rate was measured by dividing the mean number of seedlings observed by the estimated number of surviving seeds. The proportion of seeds remaining dormant was determined by dividing the number of viable seeds (tested as above) by the estimated number of surviving seeds. Again in 2005, 38 seeds cages were removed from among 13 populations to determine germination rates and dormancy of seeds at 33 months following dispersal. Finally in 2006, 31 cages were removed from among 13 populations to estimate germination rates and dormancy of seeds at 45 months following dispersal. Because seed cages were not present at all of the 18 populations, seed cage data was pooled across sites.

***Matrix population models—*** Transition probability matrices successfully summarize stage-structured life cycles like that of *P. quinquefolius* (Caswell 2001). Unique matrices (**A**) were created for populations that incorporated transition probabilities from one stage class to another over the May to May census period. Census data were combined with seed cage data to create 8 X 8 transition probability matrix models (Table 5.2). The elements in the upper left portion of the matrix represent survival within and germination from the seed bank, with the May to May transitions estimated from the seed cage data. The elements in the first row of the matrix ($a_{16}$, $a_{17}$, $a_{18}$) are fecundity values for juvenile, small adults and large adults, respectively; these values were determined by multiplying the seeds produced in August by survival rate of seeds to the following May. Seeds of each age class (9, 21 and 33 months) staying dormant to the following May are represented by elements $a_{21}$, $a_{32}$ and $a_{43,}$ respectively. Because less than one seed per population was estimated to be viable beyond 45 months, germination rate of seeds from this age class was assumed to be zero ($a_{54} = 0$). The lower right portions of the matrix are transition probabilities determined from census data. For example, element $a_{76}$ is the portion of 2-leaved juvenile plants that grew to 3-leaved small adults from one May to the next.

Population growth rate, or the finite rate of increase, is the dominant eigenvalue ($\lambda$) of **A** (Caswell 2001). For populations measured over several census periods, the product of the individual matrices represents the cumulative transition probabilities for the entire period (Caswell and Trevisan 1994, Vavrek et al. 1997, Caswell 2001). We

found a multi-year transition matrix for each population by multiplying the yearly transition matrices in MATLAB v. 7.1 (MathWorks 2005). However, the number of yearly matrices observed varied between populations, such that the growth rates were observed over different numbers of years. To standardize growth rates for the number of transitions observed, we determined

$$\lambda = \sqrt[n]{\lambda_P}$$

where $n$ equals the number of census periods from which the product matrix was calculated and $\lambda_P$ is the dominant eigenvalue from the product matrix.

**Estimation of Effective Population Size**

Effective population size ($N_e$) is a better predictor of a population's vulnerability to genetic stochasticity than the number of individuals ($N$) (Frankham 1995). $N_e$ is typically smaller than $N$ for a variety of reasons, e.g., the presence of non-reproductive individuals (Nunney and Elam 1994, Frankham 1995). Estimating $N_e$ for plants can be complex, especially for species like *P. quinquefolius* with overlapping generations and considerable variation in reproductive output (Nunney and Elam 1994, Frankham 1995). Nevertheless, we sought to obtain a population size measure reflective of $N_e$, so we used the number of reproductive individuals as an estimate of effective population size. Fluctuations in population size can make $N_e$ more resemble their smallest value than their arithmetic average (Frankham et al. 2002); thus, we used the harmonic mean of the number of reproductive plants across years

$$N = t / \Sigma(1 / N_i)$$

where $t$ is equal to the number of years and $N_i$ is the number of reproductive individuals in the $i$th year.

**Harvest Index**

Harvest is likely an infrequent event in terms of a population's history (Chapter 3) and our census period was relatively brief. We estimated the harvest pressure of each population based on its lasting impacts on stage-structure (Van der Voort et al. 2003). Following an experimental harvest of all plants, seedlings and juvenile plants made up the majority of a population for at least 5 years (Van der Voort et al. 2003). We codified harvest pressures of each of the 18 population using the mean proportion of seedlings and juvenile plants observed across the census period.

**Genetic Diversity**

Random amplified polymorphic DNA (RAPD) markers were selected because of their cost-effectiveness and applicability to uncharacterized genomes such as *P. quinquefolius* (Sunnucks 2000). Also, several previous researchers have found sufficient polymorphism and reproducibility using RAPD markers in both wild and cultivated *P. quinquefolius* (Boehm et al. 1999; Bai et al 1997; Schluter and Punja 2002; Lim 2004). Because of the caveats previous authors have noted about RAPD markers (Pérez et al. 1998, Isabel et al. 1999, Nybom and Bartish 2000), precautions were taken at multiple steps within the procedures to address these issues.

***Tissue collection and DNA extraction—*** Because diversity measures from RAPDs have shown sensitivity to small or uneven sample sizes (Isabel et al. 1999), plant material (single leaflets) was sampled from 18 randomly-selected juvenile or adult plants in all 18 populations. Seedlings were excluded from sampling under the assumption that they would be most negatively affected by leaflet removal. In the field, leaflets were placed into individual plastic bags and kept on ice in a cooler. The leaflets were divided into two portions and one half (approximately 50 mg) was manually ground in a 1.5-ml microcentrifuge tube with a micropestle. DNA was subsequently extracted from the ground tissue following the standard protocols of the Qiagen DNeasy Plant Mini Kit (Valencia, California). DNA was eluted with 100 μl of DNeasy AE buffer twice. The DNA concentration and purity of eluates was estimated spectrophotometrically using a GeneSpec I (MiraiBio, Inc) to estimate the efficacy of the kit extractions. Extractions were repeated using the remaining leaf material if concentrations were low or if samples performed poorly in subsequent reactions.

***Amplification and characterization of samples***— Because RAPDs have shown a lack of reproducibility (Pérez et al. 1998), primers were chosen that had been used with success by previous researchers in *P. quinquefolius* (Bai et al. 1997; Schluter and Punja 2002; Lim 2004). Fourteen 10mer oligonucleotide primers were obtained from the Nucleic Acid Protein Service Unit at the University of British Columbia (6, 18, 81, 98, 164, 177, 203, 210, 227, 326, 398, 419, 464 and 497). All fourteen primers were screened for reproducibility both within and between PCR reactions using five samples from each of six populations. Six primers (6, 18, 177, 203, 210, and 464) that showed

reproducible polymorphism within and between populations were chosen for RAPD PCR analysis of all 324 samples.

Reaction mixtures for RAPD PCR were assembled using the components of the Qiagen Taq PCR Core Kit (Valencia, California). Each 25 μL reaction contained 3 μg DNA (suspended in AE buffer from DNeasy kit), 2.5 μL 10 X Qiagen PCR buffer (with 15mM MgCl2), 1.0 μL 2.5mM dNTP, 0.5 μL of primer, 0.25 μL Taq polymerase and 17.75 μL sterile water. Amplifications were performed using a Bio-Rad MyCycler (Hercules, California) programmed with denaturation, annealing and extension protocols adapted from Bai et al. (1997). Following an initial 2 min denaturation step at 94°C, the reactions underwent 45 cycles of 15 s at 94°C, 45 s at 35°C and 2 min at 72°C, and then a final extension step at 72°C for 10 min.

RAPD PCR amplification products were visualized by loading 10 μL of the reaction into a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and separated by electrophoresis at 100V for 50 minutes. Following electrophoresis, banding patterns were revealed by placing each gel over UV transillumination within the AlphaImager console (Alpha Innotech, San Leandro, California) and a digital photograph was then taken using the AlphaEaseFC Imaging System software. By including a 100 bp DNA ladder (New England BioLabs) with fragments of known length in each gel, amplified loci were characterized by length using AlphaEaseFC stand alone software. Samples were scored for presence (1) or absence (0) of a band at a particular locus. The number

of scored bands per primer was pruned to include only those bands whose presence or absence could be reliably determined.

***Genetic data analysis—*** The dominant nature of RAPD markers means that the heterozygote is indistinguishable from the homozygous dominant; both possess a primer recognition site, and thus show an amplification product at a particular locus. This property precludes the direct calculation of allele frequencies and conventional population diversity statistics like Wright's (1969) F-statistics, unless Hardy-Weinberg equilibrium can be assumed (Lynch and Milligan 1994). Co-dominant allozymes have detected high levels of inbreeding in *P. quinquefolius* populations (Grubbs and Case 2004; Cruse-Sanders and Hamrick 2004), such that Hardy-Weinberg equilibrium would be unlikely. Application of Bayesian inference methods allows for estimation of F-statistics from dominant marker data (RAPDs in particular) (Holsinger 1999; Holsinger et al. 2002; Holsinger and Wallace 2004). This approach allows genetic diversity statistics to be obtained despite uncertainty about both $F_{IS}$ and Hardy-Weinberg equilibrium within populations (Holsinger et al. 2002). Using HICKORY v1.0.4 software, we were able to calculate the Bayesian equivalents of $F_{ST}$ ($\theta^B$) and $F_{IS}$ ($f$) for all polymorphic loci scored across 18 plants from 18 populations (Holsinger and Lewis 2003). In addition, we estimated the genetic diversity within each population with *hs*, average panmictic heterozygosity (Holsinger and Lewis 2003; Miller and Schaal 2006). All statistics were separately calculated using all of the models within HICKORY (full model, $f$=0, $\theta$=0, and $f$ free) using non-informative priors and the default parameters for the Monte Carlo Markov Chain sampler (Holsinger and Lewis 2003). From the four separate outputs, a

single model was chosen that minimized the Deviance Information Criterion (DIC) and dBar, a measure that accounts both for goodness of fit and the number of parameters employed to achieve the fit (Holsinger and Lewis 2003).

**Path Analysis**

We used path analysis to evaluate how harvest pressure, genetic diversity and population size influence population growth rate (Shipley 2002). In its hypothesis testing role, path analysis allows for a specific model to be developed *a priori* and tested for support using the observed data (Mitchell 1992, Petraitis et al. 1996, Shipley 2002). We constructed a path model using Amos v 5.01 (Amos Development Corp, Spring House, PA) representing the hypothesized relationship among the variables. In the model, harvest is hypothesized to have 2 direct causal relationships, represented by single-headed arrows originating from harvest index (Figure 5.1). Van der Voort and McGraw (2006) demonstrated the influence of varying harvest intensities on population growth rate. The other causal relationship from harvest index will be with effective population size; fewer reproductive plants are likely in harvested populations because they can be directly targeted through size selective harvest (Mooney and McGraw 2007). Removal of plants will also cause populations to be skewed towards smaller non-reproductive individuals (Van der Voort et al. 2003). It is through the removal of individuals that harvest leads to declines in genetic diversity of targeted populations (Cruse-Sanders et al. 2005). As observed in other plant species, small populations of *P. quinquefolius* have reduced genetic diversity relative to large populations (Grubbs and Case 2004); the model contains a causal path from our population size measure ($N_e$) to genetic diversity (*hs*),

which would quantify the consequences of inbreeding and drift. The hypothesized demographic consequences of genetic diversity are symbolized by the path from genetic diversity to population growth rate ($\lambda$). One alternative to our model could include a direct path from effective population size to population growth rate, which could represent, for example, Allée effects arising from pollen limitation (Hackney and McGraw 2000). With four paths to be estimated, our sample size (N = 18) falls below the minimum sample size of 5-20 times the number of paths suggested as a rule of thumb for path analysis (Petraitis et al. 1996). Based on the weak and non-significant correlation between $N_e$ and population growth rate ($r = 0.2290$, $p= 0.3698$) and sample size limitations, we decided to leave this relationship out of the model.

A specific covariance matrix is implied by the path model, and this can be compared to the observed covariance matrix, generally by a maximum likelihood based goodness of fit test (Petraitis et al. 1996, Shipley 2003). Depending on the particular model, sample size, and the population distribution, maximum likelihood may not be the optimal estimation technique (Byrne 2001, Shipley 2003). We compared 4 possible estimation techniques: asymptotically distribution-free (ADF), maximum likelihood (ML), generalized least squares (GLS), and scale-free least squares (SLS). For each estimation technique, the model was fit to each of 1,000 bootstrap samples and the fit assessed by four discrepancy criteria ($C_{ADF}$ , $C_{ML}$, $C_{GLS}$, and $C_{SLS}$). The estimation technique from among the four bootstrap permutations was chosen that yielded the lowest discrepancy, regardless of the discrepancy criterion considered (Arbuckle 2003).

Once the estimation technique was identified, we proceeded with the analysis. We calculated standardized path coefficients, direct and indirect effects. Standardized path coefficients give the magnitude and direction of the change in dependent variable that would occur given a one standard deviation change in the independent variable, with the contributions of the other variable held constant (Shipley 2003). Model fit was analyzed using $\chi^2$ statistics and three non $\chi^2$-distributed functions (general fit index, GFI; normal fit index, NFI; and Tucker-Lewis Index, TLI). As a relative measure of fit, these indexes describe the improvement of the model's fit compared to a null model of no association between the variables. All analyses were performed using Amos v. 5.0.1 software (Amos Development Corp 2003).

## Results

### Effective Population Size Estimates

Mean effective population sizes ranged between 4 and 166 individuals classified as reproductive across study years (Table 5.1). As expected due to variation in stage distribution, the census population size did not necessarily predict the numbers of reproductive plants. The population with the smallest mean number of reproductive plants (AD, $N_e = 4$) was not the smallest population in terms of census population size. But the largest population (P5) did have the highest mean number of reproductive plants ($N_e = 166$). Overall, 14 of 18 populations had less than half of the mean number of individuals classified as reproductive across years.

### Harvest Index

Measured as the mean proportion of seedlings and juveniles in the population, the harvest index took on a range of values across the 18 populations. Interestingly, the populations having experienced the greatest and least harvest according to this measure, VC and RD respectively, were both among the larger populations in this study. In the RD population, nearly all of the 129 individuals censused between 2003 and 2006 were either seedlings or juveniles (HI = 0.9582). At the other extreme, only 39.0% of 161 individuals censused in the VC population were seedlings or juveniles (HI = 0.3896). Beyond harvest index, two notable incidences of harvest we observed during the census period. The AD population was harvested twice, and the earliest occurred between the August 2002 and May 2003 censuses when 12 of 40 plants were harvested. Between the August 2004 and June 2005 census, 17 of 55 plants were again harvested from the AD population. Similarly, following August 2005 but prior to June 2006, 18 of 102 plants were harvested from the EB population. The harvest events at the AD population is reflected by a high harvest index (HI = 0.8638), but the harvest index was not as high for the EB population (HI = 0.5057).

**Genetic Diversity**

The 6 primers used in the RAPD analysis of 324 plants sampled from 18 populations of *P. quinquefolius* resulted in 91 amplification products of unique molecular weights (bands). Across all plant samples, a mean of 15 bands were produced by each primer (Table 5.3). Of the total number of bands produced, 57 were both reproducible (presence or absence consistent between replicate PCR reactions) and reliably scorable (presence or absence unambiguous) both within and between gels; only this set of loci

were used for diversity measures. Each primer produced between 2 to 4 monomorphic loci, i.e. bands present in all 382 plant samples. A total of 41 loci were polymorphic (presence of bands varied either within or between populations) and thus were used in the subsequent analyses.

In the analysis of population diversity statistics within HICKORY, the full model option using non-informative priors yielded the best model according to Holsinger and Lewis' (2002) model selection criteria (DIC = 1888.3, Dbar = 1551.3). From the full model analyses, we were able to obtain the Bayesian equivalents of Wright's (1969) F-statistics and average panmictic heterozygosity. For the sample of 18 individuals sampled from each of 18 populations, Wright's (1969) inbreeding coefficient ($F_{IS}$) was equal to 0.6768 (standard deviation = 0.1613), as estimated by the Bayesian equivalent *f*. Likewise, Wright's fixation index $F_{ST}$ was equal to 0.5474 (standard deviation = 0.0197), as estimated by $\theta^B$. The average panmictic heterozygosity varied from a low of 0.1058 in the CL population and a high of 0.3261 in the W4 population.

**Path Analysis**

The discrepancy criteria revealed that estimation using an asymptotically-free distribution provided a consistent fit between bootstrap samples of the data and the model ($C_{ADF}$ = 12.854, $C_{ML}$ = 16.374, $C_{GLS}$ = 13.712, $C_{ULS}$ = 14.403). Model evaluation using the $\chi^2$ statistic showed that that the null hypothesis was accepted, i.e. the covariance matrix implied by the model did not depart significantly from the observed covariance matrix (df = 2, $\chi^2$ = 1.761, p=0.415). The general fit index was close to 1 (GFI = 0.975),

indicating a good model fit, but the model fit as assessed by the normal fit index (NFI = 0.875) was less robust. The Tucker-Lewis index value was 1.114; TLI values greater than 0.90 typically indicate a good fit (Mitchell 1992).

Path coefficients varied in strength and significance depending on the relationships between variables they described (Figure 5.2). Population growth rate was negatively influenced by harvest index ($P_{HI,\lambda}$ = -0.488, p = 0.028). The majority of the total effects of harvest index on population growth rate were attributable to these direct effects (Table 5.4). The indirect effects of harvest index on population growth rate were also negative, which as modeled would occur through the influences of harvest index on effective population size, and in turn, genetic diversity. The influence of harvest index on effective population size was negative ($P_{HI,Ne}$ = -0.328, p = 0.055) and the estimated error term for effective population size ($e_1$ = 1878.06 ± 777.71) was significantly different from zero (p = 0.016). Effective population size positively influenced genetic diversity ($P_{Ne,hs}$ = 0.284, 0.023). Through the effects of harvest index on $N_e$, the standardized indirect effects of harvest index on *hs* were negative (-0.093). The estimated error term for genetic diversity was relatively small ($e_2$ = 0.003 ± 0.001) but significantly different from zero (p = 0.020). There was also a trend for population growth rate to be positively influenced by genetic diversity ($P_{hs,\lambda}$ = 0.252, p = 0.060) population size has a positive indirect effect on population growth rate, although the magnitude of this effect is relatively small (0.071). The error term for population growth rate ($e_3$ = 0.008 ± 0.007) was not significantly different from zero (p = 0.303).

## Discussion

Our research objective was to evaluate the relevance of genetic diversity identified through neutral variation to population performance in *P. quinquefolius*. The diversity statistics obtained from RAPD variation confirmed that populations of *P. quinquefolius* are likely subject to inbreeding and genetic drift. Both the estimated inbreeding coefficient ($F_{IS}$= 0.6768) and the fixation index ($F_{ST}$ = 0.5474) were large, indicating high levels of inbreeding within populations and considerable subdivision among populations. Two other studies of *P. quinquefolius* that used allozymes provide comparable results (Grubbs and Case 2004, Cruse-Sanders and Hamrick 2004). In 32 wild populations assessed by Grubbs and Case (2004), their equivalent to $F_{IS}$ was estimated to be 0.62, whereas in 21 populations studied by Cruse-Sanders and Hamrick $F_{IS}$ was estimated to 0.416. However, some recent studies have found that $F_{IS}$ generated by the Bayesian methodology is less robust than estimates of $F_{ST}$ (Holsinger and Wallace 2004, Miller and Schaal 2006). Despite this uncertainty about $F_{IS}$, our results are similar to previous studies suggesting relatively high levels of inbreeding within populations. Similarly high levels of population structuring were found with allozymes, although the authors used different measures of among population diversity (Grubbs and Case 2004, Cruse-Sanders and Hamrick 2004).

The path representing the direct effects of genetic diversity on population growth rate is of primary importance to evaluating our original research question. Although the level of significance was that of a trend, this was nevertheless observed with a relatively small sample size. Generally, the implied relationship is in agreement with the

predictions of conservation genetics theory: genetic diversity has a positive influence on population growth rate. Similar results have been found in other rare plant species when genetic variation was related to components of population growth, for example seed production (Fischer and Matthies 1998, Menges and Dolan 1998, Schmidt and Jensen 2000, Vergeer et al. 2003, Dittbrenner et al. 2005). Menges and Dolan (1998) also collected demographic data over many years and found that genetic variation was a strong predictor of population growth rate in *Silene regia*, a prairie perennial.

Interpretation of the relationship we found between genetic diversity and population growth rate relies on several important underlying assumptions. One central assumption is that our path model is appropriate. The $\chi^2$ goodness of fit test in path analysis simply says we have not rejected our model as a possibility (Mitchell 1992). As in other statistical tests, small sample sizes would increase the likelihood of false hypothesis acceptance (Sokal and Rohlf 1995). However, the relative fit measures are somewhat more resilient to small sample sizes, and they for the most part support the model. Another part of a model's appropriateness is that it does not leave out variables that could simultaneously affect diversity and population growth rate. In path analysis, variation that is not accounted for by the modeled relationships is subsumed into the error or 'unknown' variables (Shipley 2003). The path model accounts for most variation in population growth rate, but some unmodeled variation is present for genetic diversity. As a descriptive study, the possibility of unmeasured factors being responsible for the relationships we observed remains a caveat, despite the inferences available through path analysis. We also assume that the average panmictic heterozygosity measured from the

RAPD variation of 18 sampled plants gauges genome-wide variation of the entire population. In this way, average panmictic heterozygosity could reflect the results of inbreeding and genetic drift if it relates to population size. The path coefficient from effective population size and genetic diversity was positive and significant. Our results show that smaller populations have indeed retained less diversity than larger populations, supporting this part of the conservation genetics paradigm. Most of the 6 perennial species reviewed by Oostermeijer and colleagues (2003) had significant positive correlations between population size and diversity measures. In contrast, small populations of *Succisa pratensis*, a long-lived perennial, were remnant collections of more diverse adults (Vergeer et al. 2003). Another underlying assumption is that if the inbreeding depression or fixation of deleterious recessives impacted vital rates, these effects would be reflected in population growth rates. In a related study, seedlings produced by self-pollination showed reduced leaf areas in year two of growth relative to cross-pollinated seedlings (Chapter 4). Although survival of seedlings over this time frame was not affected, leaf area achieved at the seedling stage is an important predictor of longer term survival in wild populations (Chapter 4); these results suggest that inbreeding depression may affect recruitment, which is one component of population growth.

By explicitly including a harvest index in the path model, we have addressed one factor that could be simultaneously affecting population size, genetic diversity and growth rate. Based on the path coefficient, harvest index has a relatively strong negative effect on population growth rate. Any harvest occurring during the census period will

clearly affect survival rates for the affected stage classes. Recruitment may also be affected if plants are harvested prior to seed ripening or if seeds are taken off site (Van der Voort and McGraw 2006). However, harvest did not occur during the census period for most populations with relatively high harvest indexes. Because our harvest index presumably measures the 'fingerprint' of past harvest events, the relationship we found shows the lasting effects on population growth rate. These lasting effects could originate for example from seed set being limited by reduced numbers of reproductive plants in harvested populations (Hackney and McGraw 2001). The path coefficient describing the effect of harvest index on the numbers of reproductive plants suggested a negative influence of harvest index, although it was not statistically significant.

The indirect effects of harvest index on genetic diversity were negative, but relatively weak. In our model, indirect effects measure how genetic diversity changes when harvest index changes, as transmitted through effective population size (Shipley 2003). In a harvest simulation study (Cruse-Sanders et al. 2005), average within-population genetic diversity ($H_e$) declined as adult plants were removed when the post-harvest population was compared to the pre-harvest population. Populations in areas where harvesting is permitted also show reduced within-population genetic diversity relative to protected populations (Cruse-Sanders and Hamrick 2004). The direction of the related path coefficient we found was consistent with these previous results, although our measure of within-population variation was different and our harvest index was a continuous variable. With a long-lived soil seed bank, *P. quinquefolius* may also have a buffer to some extent to bottleneck effects, as is seen in other species (McCue and

Holtsford 1998). If populations have recovered numbers of reproductive adults from germination from the soil seed bank, effects of harvest on diversity would be dampened.

Our results demonstrating a positive influence of genetic diversity on population growth rate adds to the evidence in support of the importance of genetic factors in the persistence of small populations. Recent reviews have recognized the challenges and opportunities for integrating demographic and genetic data to resolve this issue underlying conservation genetics (Oostermeijer et al. 2003, Ouborg et al. 2006). However, the importance of genetic versus environmental stochasticity in determining population persistence will depend on the evolutionary history and contemporary threats, which may be species-specific. Given the breadth of wild harvest of plants worldwide (Hamilton 2004, Ticktin 2004), harvest is not a conservation issue unique to *P. quinquefolius*. We were able to assess the direct effects of harvest on population growth rate, as well as its indirect effects in terms of reduction in effective population size and genetic diversity. The results obtained for *P. quinquefolius* have implications for other commercially-collected plant species: maintenance of genetic diversity can have benefits for population performance over time frames relevant to conservation efforts.

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Table 5.1: Duration of census period and demographic data for each of the 18 study populations. Population size ($N$) is the mean number of plants observed across census years, effective population size ($N_e$) is the harmonic mean number of reproductive plants, harvest index (see description in text) and growth rates ($\lambda$) for the entire census period.

| *Population* | | *Census* | $N$ | $N_e$ | *Harvest* | $\lambda$ |
|---|---|---|---|---|---|---|
| *State* | *Name* | *Years* | | | *Index* | |
| VA | WY | 5 | 29.2 | 11.5 | 0.6516 | 0.9938 |
| WV | BN | 5 | 34.6 | 12.8 | 0.7689 | 0.9492 |
| WV | EB | 3 | 45.3 | 27.2 | 0.5057 | 0.6336 |
| VA | MT | 5 | 54.8 | 23.1 | 0.4976 | 1.0119 |
| WV | CL | 5 | 59.6 | 15.6 | 0.9131 | 0.9731 |
| NY | TP | 3 | 62.0 | 19.4 | 0.7235 | 1.2394 |
| WV | AD | 5 | 66.2 | 4.1 | 0.8638 | 0.8319 |
| WV | BS | 5 | 76.2 | 45.1 | 0.5040 | 1.0454 |
| WV | LS | 5 | 77.8 | 36.9 | 0.7480 | 1.1987 |
| WV | W2 | 5 | 101.2 | 48.1 | 0.6646 | 1.0690 |
| WV | W4 | 5 | 104.0 | 36.0 | 0.7084 | 1.1022 |
| IN | TR | 4 | 110.5 | 20.7 | 0.7802 | 1.0639 |
| WV | P4 | 5 | 125.0 | 42.0 | 0.7881 | 1.0437 |
| WV | SR | 5 | 126.8 | 90.5 | 0.4703 | 1.1056 |
| VA | RD | 3 | 128.7 | 55.1 | 0.9582 | 0.9208 |
| IN | VC | 5 | 161.4 | 105.6 | 0.3895 | 1.1698 |
| VA | PO | 4 | 259.8 | 37.7 | 0.8207 | 1.0781 |
| WV | P5 | 5 | 382.6 | 166.0 | 0.6671 | 1.0167 |

Table 5.2: Matrix of typical May to May transitions for *Panax quinquefolius*. Transitions among seeds of different ages in the soil seed bank (--- outlined) were estimated from experimental seed cages.

| | ***Seeds 9 mo*** | ***Seeds 21 mo*** | ***Seeds 33 mo*** | ***Seeds 45 mo*** | ***1-leaf*** | ***Juveniles*** | ***Small Adults*** | ***Large Adults*** |
|---|---|---|---|---|---|---|---|---|
| ***Seeds 9 mo*** | **0** | **0** | **0** | **0** | **0** | $a_{16}$ | $a_{17}$ | $a_{18}$ |
| ***Seeds 21 mo*** | $a_{21}$ | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| ***Seeds-33 mo*** | **0** | $a_{32}$ | **0** | **0** | **0** | **0** | **0** | **0** |
| ***Seeds 45 mo*** | **0** | **0** | $a_{43}$ | **0** | **0** | **0** | **0** | **0** |
| ***1-leaf*** | $a_{51}$ | $a_{52}$ | $a_{53}$ | **0** | $a_{55}$ | $a_{54}$ | **0** | **0** |
| ***Juveniles*** | **0** | **0** | **0** | **0** | $a_{65}$ | $a_{66}$ | $a_{67}$ | **0** |
| ***Small Adults*** | **0** | **0** | **0** | **0** | **0** | $a_{76}$ | $a_{77}$ | $a_{78}$ |
| ***Large Adults*** | **0** | **0** | **0** | **0** | **0** | **0** | $a_{87}$ | $a_{88}$ |

Table 5.3: List of six RAPD 10-mer primers, their sequences and descriptions of their amplification products

| *Primer* | *5' to 3' Sequence* | *Number of scorable loci* | *Number of monomorphic loci* | *Size of polymorphic loci (bp)* |
|---|---|---|---|---|
| UBC 6 | CCTGGGCCTA | 8 | 2 | 2805, 1115, 990, 860, 735, 630 |
| UBC 18 | GGGCCGTTTA | 11 | 2 | 2700, 1970, 1710, 1320, 1040, 920, 745, 640, 505 |
| UBC 177 | TCAGGCAGTC | 9 | 3 | 1620, 1240, 1145, 940, 870, 635 |
| UBC 203 | CACGGCGAGT | 11 | 2 | 1810, 1535, 1290, 1165, 1055, 945, 845, 605, 505 |
| UBC 210 | GCACCGAGAG | 9 | 3 | 2580, 1320, 1145, 970, 810, 485 |
| UBC 464 | CACAAGCCTG | 9 | 4 | 2215, 1760, 1560, 1250, 1070 |
| TOTAL | | 57 | 16 | |

Table 5.4: Decomposition of path coefficients into standardized direct, indirect and total effects of harvest index, population size ($N_e$) and genetic diversity (hs) on population growth rate (λ). The indirect and direct effects for genetic diversity (hs) are also given.

| | *Effects on population growth rate* (λ) | | |
|---|---|---|---|
| | Direct | Indirect | Total |
| Harvest index | -0.488 | -0.023 | -0.511 |
| Population size ($N_e$) | - | 0.071 | 0.071 |
| Genetic diversity (*hs*) | 0.252 | - | 0.252 |
| | *Effects on genetic diversity* (*hs*) | | |
| | Direct | Indirect | Total |
| Harvest index | - | -0.093 | -0.093 |
| Population size ($N_e$) | 0.284 | - | 0.284 |

Figure 5.1: Path model showing hypothesized relationships among four variables: harvest index, effective population size ($N_e$), genetic diversity (hs) and population growth rate ($\lambda$). Model was developed using Amos Graphics (Amos Development Corp Spring House, PA)

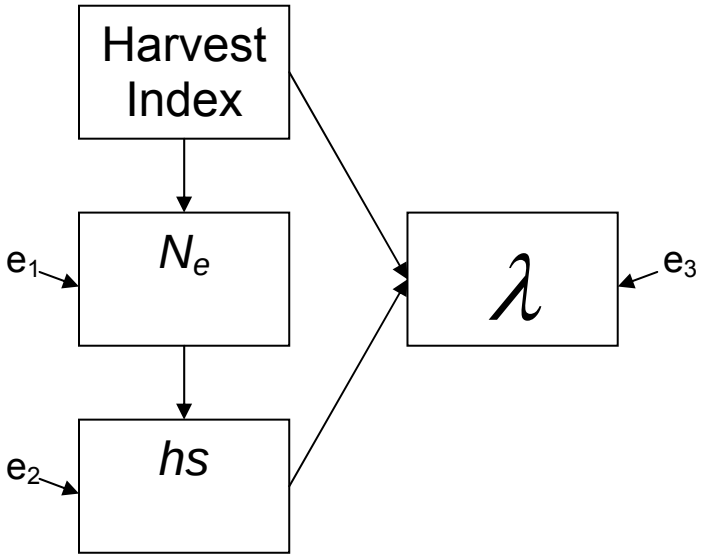
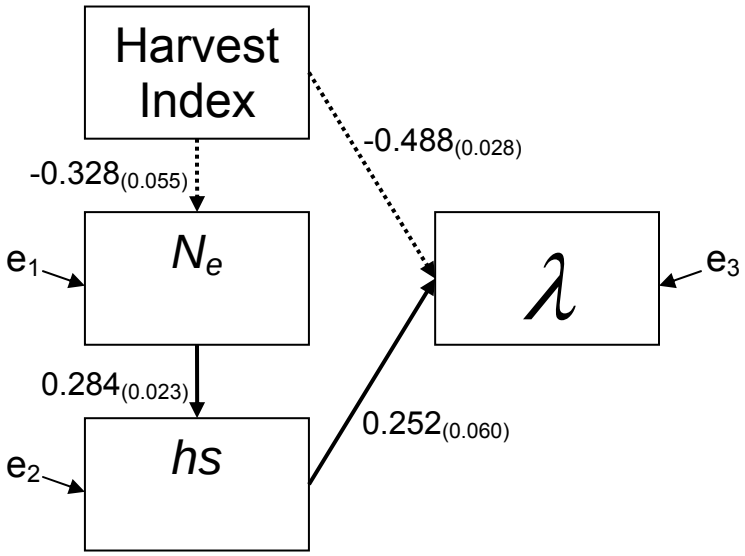


Figure 5.2: Results from path analysis; solid lines denote positive effects and dashed lines denote negative effects and associated p-values are in superscript.



# CHAPTER 6

## General Conclusions

Many plants worldwide are harvested from the wild for medicinal use, but little information is available on the sustainability of harvest or its long term impacts for most species (Hamilton 2004, Ticktin 2004, Canter et al. 2005). *Panax quinquefolius*, in this respect, is an exception: recent work has documented the consequences of harvester behavior for population persistence (Van der Voort and McGraw 2006), the impacts of harvest for genetic diversity (Grubbs and Case 2004, Cruse-Sanders and Hamrick 2005, Cruse-Sanders et al. 2006) and the relationship of harvest season timing to berry ripening (McGraw et al. 2006). However, *P. quinquefolius* is not exceptional in other ways. Throughout the world, medicinal plants can provide important sources of income to people in rural areas (Hamilton 2004), and harvest of *P. quinquefolius* serves the same purpose in the eastern United States (Bailey 1999). Many medicinal plants exist both in cultivation and the wild, for example *Piper methysticum* (kava) (Canter et al. 2005), just as *P. quinquefolius* is both an important agricultural commodity and an understory species (Suits et al. 2003). Because many aspects of its harvest are shared, results obtained from *P. quinquefolius* have implications for other wild harvested species.

My objectives were to assess some of the far-reaching genetic and evolutionary consequences of harvest for *Panax quinquefolius.* The results of the harvest simulation study revealed that humans can alter the size-fitness relationship in wild populations by selectively removing larger, more fecund plants. Altered selection from human harvest is a feature of game species: for example sport harvest of bighorn (*Ovis canadensis*) rams resulted in removal of the individuals with highest breeding success or fitness (Coltman et al. 2003). In contrast to this intentional selectivity of harvest, selection changes in *P. quinquefolius* seemed to be operating primarily through the increased apparency of larger plants to human harvesters. Similar changes

operating through apparency have been previously observed, specifically through manual weeding in cultivated settings (Barrett 1982, Kadereit and Briggs 1994). Hand weeding, for example, has resulted in varieties of the weed *Echinochloa oryzicola* that perfectly mimic rice from seedling to adult (Yamasue 2001). The harvest simulation study demonstrates that similar human-induced selection changes are possible in the wild. The fact that seed removal increased the changes to the size-fitness relationship emphasizes the importance of harvester behavior in shaping the outcomes of harvest, as noted by previous authors (Van der Voort and McGraw 2006).

Following on the size-selective mortality demonstrated by the harvest simulation, my objective was to assess any consequences for wild populations. For selection to result in evolutionary change requires that the selected traits are genetically based. Size-related traits are likely genetically based given that differences were maintained three to four years after transplantation to a common environment. I also measured the growth rates (age-size relationship) of 500 plants in 12 populations across a range harvest pressures. I found that plants of a given age were *larger* in harvested populations than plants of the same age in less harvested populations. In previous studies of human-induced evolutionary change, changes typically occurred in the opposite direction to size selectivity (Stockwell et al. 2003). In this respect, the results from the wild populations are somewhat contradictory to what would be expected from the harvest simulations. However, plants of a given age also had a greater likelihood of producing seeds in harvested populations. Similar changes have been observed as results of unintended age selection in fisheries, whereby earlier reproducing individuals have fitness advantages in harvested populations (Law 2000). Given that reproduction is size-dependent in

*P. quinquefolius*, it is possible that changes in growth rate have occurred as a result of selection against slow-growing, slow-maturing plants in harvested populations.

Previous authors have demonstrated the effects of harvest on genetic diversity using a simulation study (Cruse-Sanders et al. 2006) and by comparing diversity of harvested versus protected populations (Cruse-Sanders and Hamrick 2005). The results from both of these approaches suggested that harvest reduces genetic diversity by removing individuals. Decreases in genetic diversity are a concern in light of the potential for inbreeding depression. From the results of the controlled crosses, there was some evidence for inbreeding depression, if leaf area predicts survival as it does in wild populations. Results obtained from *P. quinquefolius* demonstrated the fitness consequences of a mixed mating system: reproductive assurance through self-pollination, but with some level of inbreeding depression (Goodwillie et al. 2005). The other intent of the controlled crosses was to evaluate the consequences of outcrossing with cultivated plants. The offspring of wild maternal plants crossed with Wisconsin pollen showed growth rates more similar to *P. quinquefolius* in cultivation than seedlings in the wild. If such rapid growth rates are conferred to later generations, wild genotypes within *P. quinquefolius* populations could be susceptible to competition from wild-cultivated hybrids, e.g., genetic swamping (Hufford and Mazer 2003). The unusual growth rates observed in the F1 suggests that cultivated seed sources are not compatible for population supplementation.

The evidence for inbreeding depression would suggest that genetic diversity is important for population performance in *P. quinquefolius;* this is a central paradigm to conservation genetics theory. I tested for support of this idea in 18 wild populations. Because of the

observational nature of this study, I chose path analysis to evaluate the relationships among diversity, effective population size and harvest pressure, and their influences on population growth rate. Although a large and diverse amount of data went into this study, a sample size of 18 populations only met the minimum requirement for robust path analysis (Petraitis et al. 1996). Nevertheless, the model was generally well supported by the data and most path coefficients were significant. Effective population size had a significant positive influence on genetic diversity; this result suggests that diversity is reduced in smaller populations, likely owing to the influences of inbreeding and drift. The impact of genetic diversity on population growth rate, although positive, was not significant. Based on these path coefficients, at least one part of the conservation genetics paradigm was supported, i.e., small populations are less diverse (Ouborg et al. 2006).

Large-scale harvest of any species from the wild clearly has many implications. Altogether, the objective of these studies was to evaluate the consequences of harvest for *P. quinquefolius* in terms of genetics and evolution. Within this intent, these studies also answered questions relevant to conservation and management of *P. quinquefolius* in the wild. Given the numbers of plants harvested from the wild worldwide, this research has implications for many other species where use or exploitation is part of their conservation circumstances.

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# Appendix

Table A.1 Age-structured seed bank transition probability matrix; these values occupied the upper left corner of every population matrix and were the came for each matrix in Chapter 5 (seed Chapter 5 Methods).

| | ***Seeds 9 mo*** | ***Seeds 21 mo*** | ***Seeds 33 mo*** | ***Seeds 45 mo*** |
|---|---|---|---|---|
| ***Seeds 9 mo*** | 0 | 0 | 0 | 0 |
| ***Seeds 21 mo*** | 0.1938 | 0 | 0 | 0 |
| ***Seeds-33 mo*** | 0 | 0.394 | 0 | 0 |
| ***Seeds 45 mo*** | 0 | 0 | 0.4125 | 0 |
| ***Seeds 9 mo*** | 0.6772 | 0.2704 | 0.375 | 0 |

Table A.2: Observed transition probability matrices for the 18 study populations used in Chapter 5. The five rows and columns correspond to the five stages: seeds, seedlings, juveniles, small adults and large adults. Transitions within the seed stage were estimated separately (See Table A.1).

Table A.2A Transition probability matrices for WY population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.2084 | 0 | 0 | 0 | 0 | 0.0834 | 0 | 0 |
| 0 | 0.6667 | 0 | 0 | 0 | 0 | 0.8571 | 0 | 0 | 0 |
| 0 | 0.3333 | 0.75 | 0.5 | 0 | 0 | 0.1429 | 0.7 | 0.4286 | 0 |
| 0 | 0 | 0.25 | 0 | 0.75 | 0 | 0 | 0.1 | 0.5714 | 1 |
| 0 | 0 | 0 | 0 | 0.25 | 0 | 0 | 0.2 | 0 | 0 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.1042 | 0.5003 | 1.5008 | 0 | 0 | 0.3207 | 0.4169 | 2.1887 |
| 0 | 0.55 | 0 | 0 | 0 | 0 | 0.6923 | 0.1538 | 0 | 0 |
| 0 | 0.15 | 0.4375 | 0.2 | 0 | 0 | 0.2308 | 0.6154 | 0.5 | 0.125 |
| 0 | 0 | 0.1875 | 0.2 | 0 | 0 | 0 | 0.2308 | 0.5 | 0 |
| 0 | 0 | 0.0625 | 0.6 | 1 | 0 | 0 | 0 | 0 | 0.875 |

Table A.2B Transition probability matrices for BN population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.0463 | 0.0926 | 0.2779 | 0 | 0 | 0 | 0.3573 | 7.0873 |
| 0 | 0.4118 | 0.0556 | 0 | 0 | 0 | 0.75 | 0.2778 | 0 | 0 |
| 0 | 0.4118 | 0.5 | 0.3333 | 0 | 0 | 0.0833 | 0.6667 | 0.2857 | 0 |
| 0 | 0 | 0.1111 | 0.3333 | 0.6667 | 0 | 0 | 0.0556 | 0.2857 | 0.5 |
| 0 | 0 | 0 | 0 | 0.3333 | 0 | 0 | 0 | 0.4286 | 0.5 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0 | 0.2084 | 0 | 0 | 0 | 0.2084 | 0.5559 | 2.0845 |
| 0 | 0.2667 | 0.1333 | 0 | 0 | 0 | 0.5556 | 0.3125 | 0 | 0 |
| 0 | 0.4 | 0.6667 | 0.5 | 0 | 0 | 0.2222 | 0.5 | 0 | 0 |
| 0 | 0 | 0.0667 | 0.5 | 0.5 | 0 | 0 | 0.1875 | 1 | 0.5 |
| 0 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0 | 0 | 0.5 |

Table A.2C Transition probability matrices for EB population

| 2004-2005 | | | | | 2005-2006 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0.8338 | 0 | 0 | 0 | 0.5956 | 1.6259 |
| 0 | 0.875 | 0.0714 | 0 | 0 | 0 | 0.0769 | 0.5625 | 0.1429 | 0 |
| 0 | 0 | 0.5714 | 0.3333 | 0 | 0 | 0 | 0 | 0.2857 | 0.05 |
| 0 | 0 | 0.2857 | 0.5 | 0.0625 | 0 | 0 | 0.1875 | 0.2857 | 0.15 |
| 0 | 0 | 0 | 0.1667 | 0.9375 | 0 | 0 | 0 | 0 | 0.3 |

Table A.2D Transition probability matrices for MT population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.0309 | 0.4548 | 0.2432 | 0 | 0 | 0 | 2.5739 | 3.0573 |
| 0 | 0.2222 | 0.037 | 0 | 0 | 0 | 0.2 | 0 | 0 | 0 |
| 0 | 0 | 0.3704 | 0 | 0.0417 | 0 | 0.2 | 0.6875 | 0.0435 | 0.1333 |
| 0 | 0 | 0.4074 | 0.4545 | 0.3333 | 0 | 0 | 0.0625 | 0.3043 | 0.2 |
| 0 | 0 | 0.0741 | 0.3636 | 0.4583 | 0 | 0 | 0.125 | 0.4783 | 0.4667 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.1563 | 0.2084 | 1.0971 | 0 | 0 | 1.0006 | 0.9096 | 3.0109 |
| 0 | 0.381 | 0 | 0 | 0 | 0 | 0.5455 | 0.1333 | 0 | 0 |
| 0 | 0.0476 | 0.5625 | 0.1667 | 0.1053 | 0 | 0.1818 | 0.4667 | 0.5455 | 0.0556 |
| 0 | 0 | 0.25 | 0.25 | 0.2632 | 0 | 0 | 0.2667 | 0.4545 | 0.2778 |
| 0 | 0 | 0.125 | 0.4167 | 0.4737 | 0 | 0 | 0.1333 | 0 | 0.6667 |

Table A.2E Transition probability matrices for CL population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0.1191 | 0 | 0 | 0 | 0.0758 | 0.4169 | 0 |
| 0 | 0.5 | 0.1111 | 0 | 0 | 0 | 0.85 | 0.2727 | 0 | 0 |
| 0 | 0.2857 | 0.8333 | 0.4286 | 0 | 0 | 0.05 | 0.5227 | 0 | 0 |
| 0 | 0 | 0.0556 | 0.5714 | 0 | 0 | 0 | 0.0227 | 0.6667 | 0 |
| 0 | 0 | 0 | 0.1191 | 0 | 0 | 0 | 0.0758 | 0.4169 | 0 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.0298 | 0 | 0 | 0 | 0 | 0.4732 | 0.3573 | 0 |
| 0 | 0.5366 | 0.1786 | 0 | 0 | 0 | 0.5 | 0.1351 | 0 | 0 |
| 0 | 0.3902 | 0.6429 | 0.6 | 0 | 0 | 0.375 | 0.7297 | 0.2857 | 0 |
| 0 | 0 | 0.1786 | 0.4 | 0 | 0 | 0 | 0.0811 | 0.7143 | 0 |
| 0 | 0 | 0.0298 | 0 | 0 | 0 | 0 | 0.4732 | 0.3573 | 0 |

Table A.2F Transition probability matrices for TP population

| 2004-2005 | | | | | 2005-2006 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0.4169 | 5.5984 | 0 | 0 | 0.0758 | 0.2084 | 6.5141 |
| 0 | 0.4667 | 0.1111 | 0 | 0 | 0 | 0.45 | 0.1818 | 0.125 | 0 |
| 0 | 0.4 | 0.5556 | 0 | 0.1429 | 0 | 0.3 | 0.7727 | 0.5 | 0.125 |
| 0 | 0 | 0.2222 | 0.5 | 0 | 0 | 0 | 0.0455 | 0.25 | 0 |
| 0 | 0 | 0.1111 | 0.5 | 0.8571 | 0 | 0 | 0 | 0.125 | 0.875 |

Table A.2G Transition probability matrices for AD population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.725 | 3.3352 | 4.169 | 0 | 0 | 0.1001 | 0.5559 | 1.5008 |
| 0 | 0.2857 | 0.1739 | 0 | 0 | 0 | 0.5424 | 0.16 | 0 | 0 |
| 0 | 0.3571 | 0.0435 | 0 | 0 | 0 | 0.0508 | 0.52 | 0.1667 | 0.2 |
| 0 | 0 | 0.087 | 0 | 0 | 0 | 0 | 0.16 | 0.3333 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.4 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.5075 | 1.2828 | 3.8911 | 0 | 0 | 0 | 0.5003 | 0 |
| 0 | 0.2712 | 0.0435 | 0 | 0 | 0 | 0.4242 | 0.25 | 0 | 0 |
| 0 | 0.0678 | 0.5217 | 0.3077 | 0 | 0 | 0.2121 | 0.4167 | 0 | 0 |
| 0 | 0 | 0.0435 | 0.1538 | 0 | 0 | 0 | 0.2083 | 0.4 | 1 |
| 0 | 0 | 0 | 0.0769 | 0 | 0 | 0 | 0.125 | 0.6 | 0 |

Table A.2H Transition probability matrices for BS population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.0758 | 0.8338 | 3.2711 | 0 | 0 | 0.1471 | 0.5095 | 3.1539 |
| 0 | 0.6429 | 0.1818 | 0 | 0 | 0 | 0.5 | 0.1176 | 0 | 0 |
| 0 | 0.2857 | 0.4545 | 0.125 | 0 | 0 | 0.1818 | 0.4706 | 0.1111 | 0.1304 |
| 0 | 0 | 0.3182 | 0.375 | 0.2692 | 0 | 0 | 0.2353 | 0.2222 | 0.3043 |
| 0 | 0 | 0.0455 | 0.375 | 0.7308 | 0 | 0 | 0 | 0.6667 | 0.5652 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.0463 | 0.0521 | 0.8659 | 0 | 0 | 0 | 0 | 0.3528 |
| 0 | 0.3333 | 0.2222 | 0 | 0 | 0 | 0.4091 | 0 | 0 | 0 |
| 0 | 0 | 0.4444 | 0.125 | 0.1538 | 0 | 0.1364 | 0.2667 | 0.3 | 0 |
| 0 | 0 | 0 | 0.5 | 0.1154 | 0 | 0 | 0.2667 | 0.3 | 0.2308 |
| 0 | 0 | 0.1111 | 0.3125 | 0.7308 | 0 | 0 | 0.4 | 0.4 | 0.7692 |

Table A.2I Transition probability matrices for LS population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.4669 | 0.0834 | 1.0422 | 0 | 0 | 0.7525 | 1.575 | 1.2507 |
| 0 | 0.625 | 0.08 | 0 | 0 | 0 | 0.5 | 0.0244 | 0 | 0 |
| 0 | 0.375 | 0.76 | 0.6 | 0.5 | 0 | 0.3333 | 0.7561 | 0.2222 | 0 |
| 0 | 0 | 0.08 | 0.1 | 0.25 | 0 | 0 | 0.1463 | 0.4444 | 0 |
| 0 | 0 | 0 | 0.1 | 0.25 | 0 | 0 | 0.0488 | 0.3333 | 1 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.5732 | 2.8046 | 3.7521 | 0 | 0 | 0.5986 | 1.6676 | 6.8175 |
| 0 | 0.5 | 0 | 0 | 0 | 0 | 0.7576 | 0.1282 | 0 | 0 |
| 0 | 0.3182 | 0.5417 | 0.2727 | 0.1667 | 0 | 0.0303 | 0.6667 | 0.4 | 0.1765 |
| 0 | 0 | 0.2083 | 0.1818 | 0.3333 | 0 | 0 | 0.0769 | 0.2 | 0.1765 |
| 0 | 0 | 0.1667 | 0.5455 | 0.5 | 0 | 0 | 0.1282 | 0.4 | 0.6471 |

Table A.2J Transition probability matrices for W2 population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.2835 | 0.3127 | 1.6676 | 0 | 0 | 0.0817 | 0.3397 | 2.5014 |
| 0 | 0.4595 | 0.02 | 0 | 0 | 0 | 0.125 | 0.0784 | 0 | 0 |
| 0 | 0.3243 | 0.62 | 0.25 | 0 | 0 | 0.25 | 0.7059 | 0.2963 | 0 |
| 0 | 0 | 0.24 | 0.625 | 0.5714 | 0 | 0 | 0.0392 | 0.2593 | 0.2857 |
| 0 | 0 | 0.06 | 0.0625 | 0.4286 | 0 | 0 | 0.0784 | 0.2963 | 0.7143 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.0596 | 0.1516 | 1.7073 | 0 | 0 | 0.0953 | 1.1749 | 3.2215 |
| 0 | 0.3043 | 0.0893 | 0 | 0 | 0 | 0.5238 | 0.1143 | 0 | 0 |
| 0 | 0.1739 | 0.4643 | 0.3636 | 0.0476 | 0 | 0.1905 | 0.6 | 0.3182 | 0.1818 |
| 0 | 0 | 0.25 | 0.3636 | 0.1905 | 0 | 0 | 0.2 | 0.4091 | 0.0455 |
| 0 | 0 | 0.1071 | 0.1818 | 0.6667 | 0 | 0 | 0.0857 | 0.2727 | 0.7727 |

Table A.2K Transition probability matrices for W4 population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.0834 | 0.2779 | 0.2779 | 0 | 0 | 0.1551 | 0.8093 | 1.3549 |
| 0 | 0.3571 | 0 | 0 | 0 | 0 | 0.7391 | 0.0465 | 0 | 0 |
| 0 | 0.4286 | 0.7 | 0.1667 | 0.3333 | 0 | 0.087 | 0.814 | 0.2941 | 0 |
| 0 | 0 | 0.3 | 0.5 | 0.6667 | 0 | 0 | 0.093 | 0.3824 | 0.375 |
| 0 | 0 | 0 | 0.1667 | 0 | 0 | 0 | 0.0233 | 0.2059 | 0.5 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.6014 | 1.9455 | 3.2035 | 0 | 0 | 0.2084 | 0.4548 | 1.0262 |
| 0 | 0.6047 | 0.0328 | 0 | 0 | 0 | 0.6341 | 0.0385 | 0 | 0 |
| 0 | 0.093 | 0.6885 | 0.25 | 0.0526 | 0 | 0.0976 | 0.7692 | 0.5455 | 0.1538 |
| 0 | 0 | 0.1639 | 0.5833 | 0.0526 | 0 | 0 | 0.0577 | 0.4091 | 0 |
| 0 | 0 | 0.1148 | 0.1667 | 0.8947 | 0 | 0 | 0.1154 | 0.0455 | 0.8462 |

Table A.2L Transition probability matrices for TR population

| 2003-2004 | | | | | 2004-2005 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.3848 | 0 | 3.411 | 0 | 0 | 0.1883 | 0.4632 | 1.3776 |
| 0 | 0.5882 | 0.1538 | 0 | 0 | 0 | 0.481 | 0.0323 | 0.1111 | 0 |
| 0 | 0.1176 | 0.3846 | 0 | 0.0909 | 0 | 0.1519 | 0.2903 | 0.2222 | 0.0435 |
| 0 | 0 | 0.1538 | 0 | 0 | 0 | 0 | 0.1935 | 0.2222 | 0.1304 |
| 0 | 0 | 0.3077 | 1 | 0.7273 | 0 | 0 | 0.1613 | 0.2222 | 0.6522 |
| 2005-2006 | | | | | | | | | |
| 0 | 0 | 0.1962 | 2.3346 | 2.274 | | | | | |
| 0 | 0.4925 | 0.1471 | 0 | 0 | | | | | |
| 0 | 0.194 | 0.5294 | 0.6 | 0.2727 | | | | | |
| 0 | 0 | 0.1765 | 0.2 | 0.0909 | | | | | |
| 0 | 0 | 0.1471 | 0.2 | 0.5909 | | | | | |

Table A.2M Transition probability matrices for P4 population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.0817 | 0.3752 | 0 | 0 | 0 | 0.0315 | 0.397 | 1.3341 |
| 0 | 0.4655 | 0.098 | 0 | 0 | 0 | 0.6481 | 0.2642 | 0 | 0 |
| 0 | 0.2069 | 0.6275 | 0.15 | 0.1818 | 0 | 0.1481 | 0.6038 | 0.5714 | 0 |
| 0 | 0 | 0.1176 | 0.65 | 0.3636 | 0 | 0 | 0.0189 | 0.2381 | 0 |
| 0 | 0 | 0 | 0 | 0.2727 | 0 | 0 | 0.0755 | 0.1429 | 0.8 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.3184 | 2.0845 | 1.7434 | 0 | 0 | 0.5232 | 0.7296 | 3.2035 |
| 0 | 0.5645 | 0.1455 | 0 | 0 | 0 | 0.4583 | 0.0588 | 0 | 0 |
| 0 | 0.2581 | 0.5636 | 0.3333 | 0 | 0 | 0.3125 | 0.5882 | 0.625 | 0.2105 |
| 0 | 0 | 0.1091 | 0.5 | 0 | 0 | 0 | 0.3529 | 0.375 | 0.2105 |
| 0 | 0 | 0.1455 | 0.1667 | 0.9091 | 0 | 0 | 0 | 0 | 0.5789 |

Table A.15 Transition probability matrices for SR population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2581 | 0.7357 | 0.9439 |
| 0 | 0.4091 | 0.0328 | 0 | 0 | 0 | 0.2308 | 0.0238 | 0 | 0 |
| 0 | 0.5 | 0.4754 | 0.0435 | 0.0417 | 0 | 0.4615 | 0.6429 | 0.6471 | 0.0755 |
| 0 | 0 | 0.1967 | 0.0435 | 0.125 | 0 | 0 | 0.1905 | 0.1765 | 0.1509 |
| 0 | 0 | 0.2623 | 0.8261 | 0.75 | 0 | 0 | 0.0714 | 0.1765 | 0.7358 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.1308 | 0.7147 | 3.8967 | 0 | 0 | 0.0232 | 0.0481 | 0.7437 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0.7 | 0 | 0 | 0 |
| 0 | 0.8 | 0.451 | 0.2381 | 0.0408 | 0 | 0.0333 | 0.4722 | 0.25 | 0.027 |
| 0 | 0 | 0.4314 | 0.6667 | 0.3265 | 0 | 0 | 0.0278 | 0.0577 | 0.027 |
| 0 | 0 | 0.098 | 0.0476 | 0.6327 | 0 | 0 | 0.5 | 0.6923 | 0.9459 |

Table A.16 Transition probability matrices for RD population

| 2004-2005 | | | | | 2005-2006 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0.0685 | 0 | 0 | 0 | 0 | 0.0656 | 0 | 1.0262 |
| 0 | 0.4423 | 0.0959 | 0 | 0 | 0 | 0.4878 | 0.1236 | 0 | 0 |
| 0 | 0.3654 | 0.6712 | 0.5 | 0 | 0 | 0.3171 | 0.809 | 0.8333 | 0 |
| 0 | 0 | 0.1096 | 0.25 | 0 | 0 | 0 | 0 | 0.1667 | 0 |
| 0 | 0 | 0.0685 | 0 | 0 | 0 | 0 | 0.0656 | 0 | 0.8462 |

Table A.17 Transition probability matrices for VC population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0.2036 | 0 | 0 | 0.3032 | 0.5685 | 4.4072 |
| 0 | 0.5909 | 0.04 | 0 | 0 | 0 | 0.6429 | 0 | 0 | 0 |
| 0 | 0.0909 | 0.44 | 0.0667 | 0.0349 | 0 | 0.1429 | 0.3939 | 0.3409 | 0.0571 |
| 0 | 0 | 0.12 | 0.6 | 0.3372 | 0 | 0 | 0.4242 | 0.5455 | 0.4143 |
| 0 | 0 | 0.24 | 0.2667 | 0.5698 | 0 | 0 | 0.0303 | 0.0455 | 0.4857 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.0208 | 0.0457 | 0.5559 | 0 | 0 | 0.6856 | 0.9901 | 5.2656 |
| 0 | 0.4595 | 0.05 | 0 | 0 | 0 | 0.6452 | 0.0222 | 0 | 0 |
| 0 | 0.3514 | 0.475 | 0.1233 | 0.0833 | 0 | 0.1935 | 0.7333 | 0.125 | 0.0978 |
| 0 | 0 | 0.125 | 0.1918 | 0.0278 | 0 | 0 | 0.0222 | 0.25 | 0.0217 |
| 0 | 0 | 0.25 | 0.6712 | 0.8889 | 0 | 0 | 0.2222 | 0.625 | 0.8804 |

Table A.18 Transition probability matrices for PO population

| 2003-2004 | | | | | 2004-2005 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 2.3346 | 0 | 0 | 0.0545 | 0 | 0.3254 |
| 0 | 0.4894 | 0.0976 | 0 | 0 | 0 | 0.4426 | 0.0754 | 0 | 0 |
| 0 | 0.4043 | 0.8171 | 0.5 | 0 | 0 | 0.4098 | 0.6884 | 1 | 0.2683 |
| 0 | 0 | 0.0244 | 0.5 | 0 | 0 | 0 | 0.0402 | 0 | 0.122 |
| 0 | 0 | 0.0244 | 0 | 1 | 0 | 0 | 0.1608 | 0 | 0.6098 |

| 2005-2006 | | | | |
|---|---|---|---|---|
| 0 | 0 | 0.0667 | 0 | 0.3827 |
| 0 | 0.4821 | 0.05 | 0 | 0 |
| 0 | 0.3214 | 0.745 | 0.5833 | 0.2787 |
| 0 | 0 | 0.055 | 0.0833 | 0.1148 |
| 0 | 0 | 0.14 | 0.3333 | 0.6066 |

Table A.19 Transition probability matrices for P5 population

| 2002-2003 | | | | | 2003-2004 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0.0513 | 0.3733 | 0 | 0 | 0 | 0.3088 | 2.1982 |
| 0 | 0.6525 | 0.1212 | 0 | 0 | 0 | 0.6781 | 0.2231 | 0 | 0 |
| 0 | 0.1441 | 0.553 | 0.1231 | 0.0896 | 0 | 0.137 | 0.6529 | 0.284 | 0.0303 |
| 0 | 0 | 0.1364 | 0.6 | 0.1791 | 0 | 0 | 0.0744 | 0.4321 | 0.2273 |
| 0 | 0 | 0.0303 | 0.1538 | 0.6866 | 0 | 0 | 0 | 0.2469 | 0.7273 |
| 2004-2005 | | | | | 2005-2006 | | | | |
| 0 | 0 | 0.0505 | 0.1869 | 1.2262 | 0 | 0 | 0.1131 | 0.3111 | 1.5832 |
| 0 | 0.6258 | 0.1061 | 0 | 0 | 0 | 0.6242 | 0.2034 | 0 | 0 |
| 0 | 0.2065 | 0.5682 | 0.2069 | 0 | 0 | 0.2357 | 0.6271 | 0.2687 | 0.0506 |
| 0 | 0 | 0.1894 | 0.5345 | 0.1324 | 0 | 0 | 0.1695 | 0.6716 | 0.443 |
| 0 | 0 | 0.0606 | 0.2414 | 0.8235 | 0 | 0 | 0 | 0.0597 | 0.5063 |